Insights into stimulus perception, target gene expression and network formation of LytS/LytTR-like histidine kinase/response regulator systems in *Escherichia coli*

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Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde. Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die folgende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, 08.11.2012

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I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources. As well I declare, that I have not submitted a dissertation without success and not passed the oral exam. The present dissertation (neither the entire dissertation nor parts) has not been presented to another examination board.

Munich, 08.11.2012

Contents

Nomenclature

Gene products are numbered in a way that the first methionine/valine of the wild-type protein is designated “1” in the amino acid sequence (if present: independently of the N-terminal affinity tag). N-terminal and C-terminal affinity tags are marked in genes and proteins corresponding to their position (e.g. 6His-YehT or YehT-6His).

Amino acid substitutions in proteins are termed as follows: The native amino acid is designated in one-letter code, followed by the respective amino acid position in the protein. The amino acid introduced by (site-directed) mutagenesis is terminally added in one-letter code (Example: YehT-D54E).

Deletions of genes are marked by “Δ”.

Unless otherwise noted, nucleotide positions indicate the distance from the transcriptional start site (+1).

Abbreviations

ATP	adenosine-5'-triphosphate
CA	catalytic and ATP binding domain
cAMP	cyclic adenosine-5'-monophosphate
c-di-GMP	bis-(3'-5')-cyclic dimeric guanosine monophosphate
cGMP	cyclic guanosine-5'-monophosphate
CM	cytoplasmic membrane
CP	cytoplasm
CRP	cAMP receptor protein
DHp	dimerization and histidine phosphotransfer domain
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EMSA	electrophoretic mobility shift assay
EPR	electron paramagnetic resonance
GAF	protein domain present in cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA proteins
HAMP	protein domain present in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and some phosphatases
HK	histidine kinase
n-His tag	affinity tag composed of n histidine residues
HPt	His-containing phosphotransfer protein
LB	lysogeny broth
MFS	major facilitator superfamily of transporters
RR	response regulator
PAGE	polyacrylamide gel electrophoresis
PAS	protein domain present in Per, Arnt, Sim proteins
PP	periplasm
RNase	ribonuclease
TM	transmembrane domain
TCS	two-component system
Usp	universal stress protein

Publications and Manuscripts Originating from this Thesis

CHAPTER 2

Fried, L.*, Lassak, J*., and Jung, K. (2012). A comprehensive toolbox for the rapid construction of *lacZ* fusion reporters. *J. Microbiol. Methods.* 91: 537-543.

CHAPTER 3

Kraxenberger, T.*, Fried, L.*, Behr, S., and Jung, K. (2012). First insights into the unexplored two-component system YehU/YehT in *Escherichia coli*. *J. Bacteriol.* 194(16): 4272-4284.

CHAPTER 4

Fried, L.*, Behr, S.*, and Jung, K. (2013). Identification of a target gene and activating stimulus for the YpdA/YpdB histidine kinase/response regulator system in *Escherichia coli*. 195(4): 807-815.

CHAPTER 5

Behr, S.*, Fried, L.*, Lorenz, N., and Jung, K. (2013). Identification of the LytS/LytTR-like signaling network in *Escherichia coli*. *Manuscript.*

CHAPTER 6

Jung, K., Fried, L., Behr, S., and Heermann, R. (2012). Histidine kinases and response regulators in networks. *Curr. Opin. Microbiol.*15(2): 118-124.

Contributions to Publications and Manuscripts presented in this Thesis

CHAPTER 2

L. Fried, J. Lassak, and K. Jung designed the concept of the study. L. Fried constructed the strains and performed all expression experiments. J. Lassak constructed all plasmids. L. Fried, J. Lassak and K. Jung discussed the results and wrote the manuscript.

CHAPTER 3

T. Kraxenberger, L. Fried, S. Behr, and K. Jung developed the concept of the study. T. Kraxenberger and L. Fried constructed all strains and plasmids and prepared the samples for the microarray analysis. L. Fried performed expression analysis via Northern blot. T. Kraxenberger and S. Behr determined YehT DNA-binding affinity and binding areas via EMSA, DNase I footprinting and expression analysis. L. Fried performed the initial *yjiY* expression screen and characterized the CRP binding site. T. Kraxenberger, L. Fried, S. Behr, and K. Jung wrote the manuscript.

CHAPTER 4

L. Fried, S. Behr, and K. Jung designed the concept of the study. L. Fried and S. Behr constructed all strains and plasmids and prepared the samples for the microarray analysis. L. Fried performed expression analysis via Northern blot. L. Fried and S. Behr determined YpdB DNA-binding affinity and binding areas via EMSA, DNase I footprinting and expression analysis. L. Fried performed the initial *yhjX* expression screen. L. Fried, S. Behr, and K. Jung wrote the manuscript.

CHAPTER 5

S. Behr, L. Fried, and K. Jung developed the concept of the study. S. Behr and L. Fried constructed all strains and plasmids. L. Fried performed expression analysis via qRT-PCR. S. Behr determined in vivo and in vitro protein-protein interactions. L. Fried and N. Lorenz performed the luciferase based expression screen. S. Behr and L. Fried wrote the manuscript.

CHAPTER 6

K. Jung, L. Fried, S. Behr, and R. Heermann designed and discussed the concept of the review and wrote sections 9 to 11. K. Jung wrote sections 6 and 7. L. Fried wrote section 3. S. Behr wrote sections 5 and 8 and R. Heermann wrote sections 1, 2 and 4.

We hereby confirm the above statements:

Luitpold Fried

Prof. Dr. Kirsten Jung

Dr. Jürgen Lassak

Dr. Tobias Kraxenberger

Stefan Behr

Summary

Signal transduction systems that perceive external signals are essential in maintaining intracellular processes since they induce appropriate cellular responses. Two-component systems, composed of a histidine kinase and a response regulator, are the major bacterial signal transduction systems. Among these systems, the widespread LytS/LytTR family regulates essential cellular functions in pathogenic Gram-positive bacteria. In Gram-negative bacteria, little is known about the corresponding systems.

The main aim of this thesis was to elucidate the signaling mechanisms implemented by the LytS/LytTR-like two-component systems YehU/YehT and YpdA/YpdB in the Gram-negative bacterium *Escherichia coli*. By combining genetic, biochemical and bioinformatic approaches, new insights into the relationship between signal perception, signal transduction and the generation of a cellular response were gained.

In the first study of this thesis, a functional toolbox for the generation of plasmid- or chromosomally-encoded *lacZ* reporter gene fusions is presented. The functionality of the introduced methods was demonstrated by the generation of reporter gene fusions and subsequent in vivo expression studies.

The YehU/YehT system was characterized in the second study. Transcriptome analysis, subsequent expression studies and gel retardation experiments identified *yjiY* as the target of YehT. The YehT-binding site, composed of two direct repeats of the motif, ACC(G/A)CT(C/T)A, separated by a 13-bp spacer in the *yjiY* promoter, was identified. Moreover, induction of *yjiY*, which encodes a membrane-integrated transport protein of unknown function, was demonstrated in media containing peptides or amino acids as carbon sources. Additionally, *yjiY* was induced during the mid-exponential growth phase and to be dependent on cAMP/CRP regulation.

The stimulus and the target gene of the YpdA/YpdB signal transduction system were elucidated in the third study. Again using a combination of transcriptome analysis, subsequent expression studies and gel retardation experiments, *yhjX* was identified as the target of YpdB. The YpdB-binding site was identified as well, consisting of two direct repeats of the motif GGCATTTCAT separated by a 11-bp spacer in the *yhjX* promoter. Furthermore, a comprehensive *yhjX* expression study determined that extracellular pyruvate stimulates the YpdA/YpdB-system. YhjX, a putative major-facilitator-superfamily transporter, was produced and integrated into the membrane.

The complex LytS/LytTR-like signaling network was described in the fourth study. Here, it was shown that *yjiY*, *yhjX* and *yehS* are expressed in a coordinated manner. Further, protein-protein interaction studies demonstrated that YehS, a putative accessory

protein, interacts in vivo and in vitro with the YehU/YehT and YpdA/YpdB signaling cascade. Moreover, carbon storage regulator A was found to regulate *yjiY* and *yhjX* mRNA levels post-transcriptionally.

Finally, networks between histidine kinases and response regulators are reviewed, and models are presented on how HKs and RRs employ accessory proteins to mediate signal integration, scaffolding, interconnection and allosteric regulation. Several examples are included to illustrate that TCS networks are important hubs that help to regulate the flow of cellular information.

Zusammenfassung

Eine Grundvoraussetzung für das Überleben von Bakterien ist die Wahrnehmung des sie umgebenden Milieus. Signaltransduktionssysteme erkennen extrazelluläre Reize und generieren eine angemessene zelluläre Antwort. Zwei-Komponenten-Systeme, bestehend aus einer Sensor-Histidinkinase und einem Antwortregulator-Protein, spielen eine zentrale Rolle bei der bakteriellen Signaltransduktion. Die Klasse LytS/LytTR-artiger Zwei-Komponenten-Syteme reguliert in pathogenen Gram-positiven Bakterien essentielle zelluläre Funktionen. In Gram-negativen Bakterien hingegen sind LytS/LytTR-artige Systeme nur wenig erforscht.

Im Rahmen dieser Dissertation wurden die LytS/LytTR-artigen Zwei-Komponenten-Systeme YehU/YehT und YpdA/YpdB aus *Escherichia coli* untersucht. Eine Kombination genetischer, biochemischer und bioinformatischer Methoden gewährte neue Einblicke in den Zusammenhang zwischen Reizwahrnehmung, Signaltransduktion und der daraus resultierenden zellulären Antwort.

In der ersten Studie dieser Arbeit werden Methoden zur Konstruktion von Plasmid- oder chromosomalkodierten *lacZ* Reportergenfusionen präsentiert. Die Funktionalität der Methodik wurde durch die Konstruktion von Reportergenfusionen und anschließenden Expressionsanalysen bestätigt.

Eine Charakterisierung des YehU/YehT Systems wurde im Rahmen der zweiten Studie durchgeführt. Globale Transkriptomanalysen, Expressionsstudien und Gel-Retardationsexperimente identifizierten *yjiY* als direktes Zielgen von YehT. Die YehT-Bindestelle wurde als direkte Wiederholung des Motivs ACC(G/A)CT(C/T)A, getrennt durch ein 13-bp *spacer*-Motiv, im *yjiY* Promotor identifiziert. Das Gen *yjiY*, kodierend für ein Membran-integriertes Transportprotein unbekannter Funktion, wurde in Medien mit Peptiden oder Aminosäuren als Kohlenstoffquellen exprimiert. Diese *yjiY* Induktion wurde zusätzlich durch das cAMP/CRP System reguliert.

Der Stimulus und das Zielgen des YpdA/YpdB Signaltransduktionssytems wurden im Rahmen der dritten Studie aufgeklärt. Die Kombination von Transkriptomanalysen, nachfolgenden Expressionsstudien und Gel-Retardationsexperimenten identifizierte *yhjX* als direktes Zielgen von YpdB. Die YpdB-Bindestelle wurde als direkte Wiederholung des Motivs GGCATTTCAT, getrennt durch eine 11-bp *spacer* Sequenz, im *yhjX* Promotor identifiziert. Extrazelluläres Pyruvat wurde durch Expressionsanalysen als Stimulus für das YpdA/YpdB System nachgewiesen. Zusätzlich wurde die Membran-Integration von YhjX, einem putativen Transporter der Major-Facilitator-Superfamilie, bestätigt.

In der vierten Studie dieser Arbeit wurde das komplexe LytS/LytTR-artige Signaltransduktionsnetzwerk charakterisiert. Eine koordinierte Expression der Gene *yjiY*, *yhjX* und *yehS* wurde nachgewiesen. Protein-Protein-Interaktionsstudien zeigten, dass YehS, ein putatives akzessorisches Protein, in vivo und in vitro mit den YehU/YehT und YpdA/YpdB Signaltransduktionssystemen interagiert. Die *yjiY* und *yhjX* mRNA Niveaus wurden posttranskriptionell durch den globalen Kohlenstoffregulator (*carbon storage regulator* A) CsrA beeinflusst.

In der letzten Studie dieser Dissertation wurden Histidinkinase/Antwortregulator-Netzwerke beschrieben. Prinzipien, wie akzessorische Proteine in Netzwerke integriert sind, um Prozesse wie Signal-Integration, *scaffolding*, Kopplung und allosterische Regulation zu vermitteln, werden dargestellt. Einige Beispiele zeigen, dass solche Netzwerke wichtige Schnittstellen des zellulären Informationsflusses sind.

1 Introduction

In their natural habitats, all living organisms have to adapt to changing environmental conditions in order to survive. As an evolutionary consequence, all organisms have developed specialized mechanisms to continue thriving. This is especially true for prokaryotes, the smallest living organisms that have to respond to slight changes in physical and chemical parameters such as temperature, osmolarity, oxygen content, pH, nutrient supply, and the presence of harmful substances. As prokaryotes are single cell organisms, environmental alterations directly affect the cell and its life-sustaining processes such as metabolism, transcription and translation. In order to counter life-threatening stresses, prokaryotes depend on signal/response systems (Boor, 2006). A rapid cellular response to salt and acidic stress (Heermann & Jung, 2010a, Haneburger *et al.*, 2012), heat shock (Schumann, 2012), and C limitation (Britos *et al.*, 2011, Matin, 1991) is also essential. Furthermore, recognition of other organisms from similar or different pro- and eukaryotic species is crucial. Thus, through adequate signal processing, synchronized processes such as biofilm formation (Njoroge & Sperandio, 2009), bioluminescence (Anetzberger *et al.*, 2012), and the expression of virulence factors (Rumbaugh *et al.*, 2009), can be achieved.
Typically, prokaryotic signal transduction systems consist of transmembrane (TM) proteins which sense an extracellular stimulus and transduce it into an intracellular response. Generally, three different types of signaling mechanisms are described, involving σ factors, one-component and two-component systems. One-component systems represent the simplest and predominant form. Here, the input and output domain are combined within one protein (Ulrich *et al.*, 2005). In contrast, two-component systems (TCSs) consist of at least two proteins: an often membrane-anchored histidine kinase (HK, hereinafter also referred to as “sensor kinase”), and a cytoplasmic response regulator (RR) (Mascher *et al.*, 2006, Stock *et al.*, 2000).

1.1 Two-component systems

In 1,420 bacterial genomes, 87,173 TCSs were identified (Ulrich & Zhulin, 2010). The average bacterium employs 10–50 of these systems to sense environmental conditions (Szurmant *et al.*, 2007). A correlation between the number of TCSs and the genome size was observed, with larger genomes tending to encode more TCSs (Beier & Gross, 2006). However, the number of TCS proteins differs significantly, ranging from 0 in *Mycoplasma genitalium,* 9 in *Haemophilus influenza,* 11 in *Helicobacter pylori,* 19 in *Thermotoga maritime,*

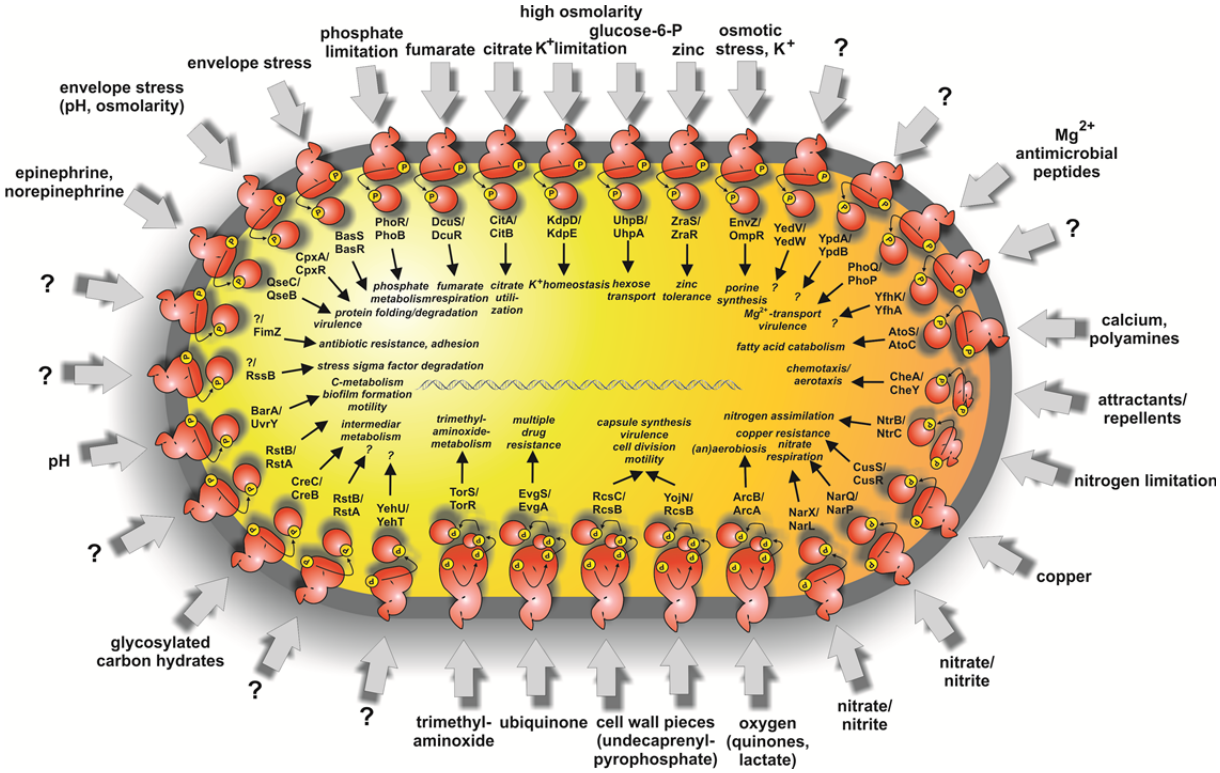

Fig. 1.1 Two-component systems of *Escherichia coli.* All membrane-anchored and cytoplasmic histidine kinases (HK) and cytoplasmic regulators are illustrated. The perceived stimuli (if known) and the generated output response are indicated. The activating stimulus, the specific HK of FimZ and RssB and the generated response of some systems are unknown (?). The figure was provided by Ralf Heermann, adapted and modified.

70 in *B. subtilis*, 80 in *Synechocystis* sp., 164 in *Streptomyces coelicolor,* 211 in *Anabaena* sp., to 251 in *Myxococcus xanthus* (Heermann & Jung, 2010b). In *E. coli*, the most thoroughly investigated prokaryote, 30 HKs and 32 RRs (of which 29 are DNA-binding) are described (Mizuno, 1997). Many TCSs are studied extensively, such as the osmotic stress- and potassium-sensing KdpD/KdpE systems (Heermann & Jung, 2010a), the magnesium-sensing PhoP/PhoQ system (Minagawa *et al.*, 2003), and the quorum-sensing system QseC/QseB (Njoroge & Sperandio, 2012). All TCSs of *E. coli* are depicted in Figure 1.1, including the perceived stimuli (if known) and the cellular response.

1.2 Stimulus perception and signaling by histidine kinases

A prototypical TCS consists of a HK, comprising the input domain and the kinase core, and a RR, containing receiver and effector domain (Fig. 1.2). Extracellular stimuli are perceived by, and serve to modulate the activities of the HK. System-specific stimulus perception by the N-terminal, membrane-integrated, input domain results in a high diversity of this domain in all HKs. The C-terminal transmitter domain consists of a dimerization and histidine phosphotransfer domain (DHp; PFAM nomenclature: His Kinase A) with a conserved histidine residue for phosphorylation and the catalytic and ATP-binding domain (CA; PFAM nomenclature: HATPase_c), which harbors the catalytic activity of transferring the

phosphoryl group from ATP to the histidine residue (Gao & Stock, 2009). Transmitter domains of all HKs share unique sequence motifs (Fig. 1.2 lower panel). The H box-containing DHp domain with the conserved histidine residue for phosphorylation mediates dimerization. Most HKs form homodimers (Ashenberg *et al.*, 2011). CA domains are characterized by N, G1, F, and G2 boxes, which are essential elements of the ATP binding site (Stewart, 2010). In general, HKs have three enzymatic activities: (auto-)kinase-, phosphotransferase- and phosphatase-activity. Stimulus perception by the input domain of the HK causes conformational changes, resulting in a dimerization of two HK molecules via the DHp domain. Subsequently, the bound ATP is hydrolyzed, followed by phosphorylation of the conserved histidine residue with the γ-phosphoryl group of the ATP (Levit *et al.*, 1996, Surette *et al.*, 1996), resulting in a high-energy phosphoamidate. Subsequently, the phosphoryl group is transferred to a conserved aspartate residue of the RR (phosphoanhydride), causing an activation of the effector domain. Signal termination is achieved by the intrinsic phosphatase activity of the HK and/or the half-life of the phosphoanhydride (s-min) (Stock *et al.*, 2000, Heermann & Jung, 2010b).

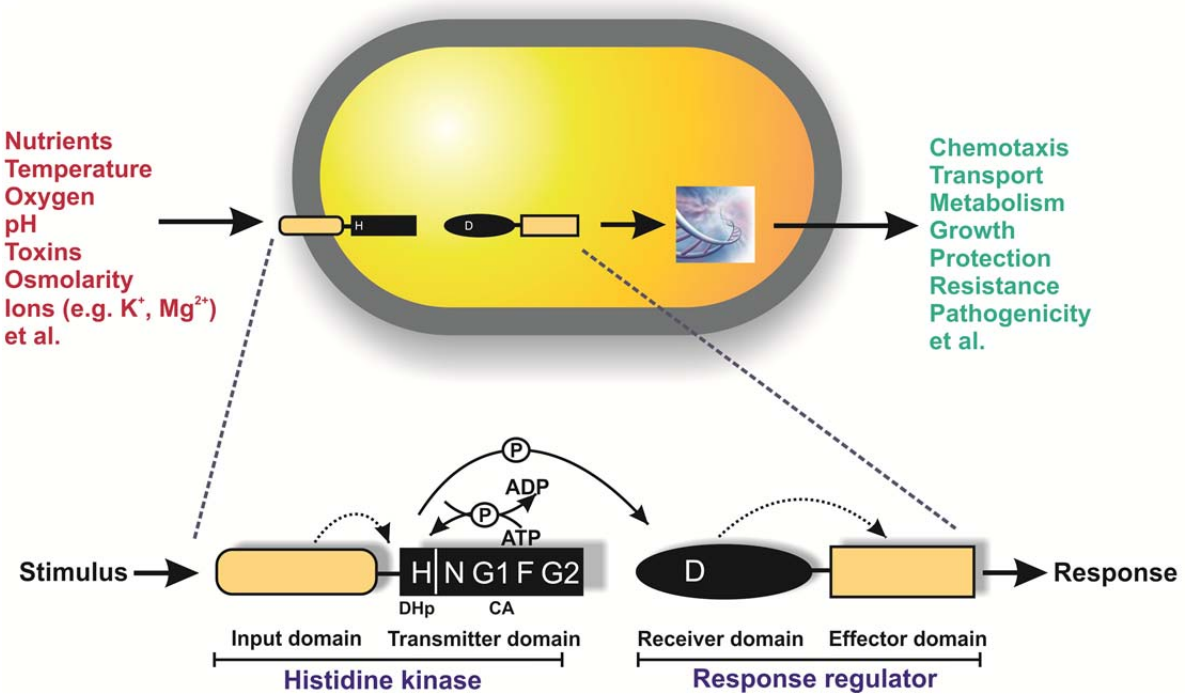

Fig. 1.2 Signaling and domain organization in prototypical two-component systems. Histidine kinases (HKs) are the input components, designated to sense the respective stimuli and correspondingly regulate the signaling pathway. Upon stimulus perception, the HK (after dimerization) autophosphorylates at a conserved histidine residue (H) mostly in trans. Subsequently, the phosphoryl group is transferred to a conserved aspartate residue (D) of the response regulator (RR), inducing a conformational change that activates the RR and triggers the cellular response. RRs, characterized by a receiver domain linked to an effector domain, mediate the output response upon activation. The figure was provided by Ralf Heermann, adapted and modified.

All HKs and RRs share a modular organization (Parkinson & Kofoid, 1992, Swanson *et al.*, 1994). Transmitter and receiver domains contain the conserved H (histidine) and D (asparate) boxes, respectively, and communicate via phosphorylation and dephosphorylation reactions (see above and Fig. 1.2). These domains are associated with various domains that function as input and output elements and are specific for each system. The input domain of a HK modulates the activity of the transmitter domain, whereas the receiver domain regulates the activity of its corresponding output domain (Heermann & Jung, 2010b).

Besides the single-step phosphotransfer, more complex systems exist, employing multiple phosphorylation steps: phosphorelay systems possess two additional components in the phosphorylation cascade; a regulator protein with a conserved aspartate residue and a His-containing phosphotransfer (HPt) protein (Hoch, 2000). Such complex systems, like the sporulation phosphorelay of *B. subtilis*, allow multiple signaling inputs/outputs, improved fine-tuning, and better cross connections (Hoch & Varughese, 2001). If downstream domains are fused to the kinase domain of the HK in one protein, they are called hybrid histidine kinases. Several phospho-donating and phospho-accepting residues in one HK can also result in a multi-stage phosphorelay. The sensing of three different types of autoinducers by the Lux-system of *Vibrio* species, is controlled by phosphorelay systems as well (Anetzberger *et al*., 2012).

In general, based on the functional mechanism of stimulus perception and domain architecture, HKs can be classified into three subgroups: periplasmic/extracellular sensing HKs (prototype: EnvZ/PhoA/VirA-like HKs), membrane sensing HKs (e.g. LiaS/BceS, LuxN, 5 TM Lyt-like HKs), and cytoplasmic sensing HKs (e.g. KdpD, ArcB) (Mascher *et al*., 2006). Stimulus perception and signal transduction in HKs can be amplified by additional sensory or linker domains, like HAMP (present in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and some phosphatases), PAS (present in Per, Arnt, Sim proteins), or GAF domains (present in cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA proteins) (Mascher *et al*., 2006, Stewart, 2010, Galperin *et al.*, 2001). HAMP domains convert sensory inputs to output response signals (Parkinson, 2010). PAS domains (Taylor & Zhulin, 1999), Usp (universal stress protein) domains (Heermann *et al.*, 2009) and GAF domains (Cann, 2007) are accessory domains for stimulus perception and signal transduction.

1.3 Structural and functional properties of response regulators

The domain architecture of response regulators is mostly characterized by a modular design of N-terminal receiver domains linked to C-terminal effector domains (Fig. 1.2 lower panel). Sixteen percent of all RRs harbor a single receiver domain. So far, 98,520 of RR sequences have been predicted (Ulrich & Zhulin, 2010), and 200 proteins have been structurally and/or functionally characterized (Bourret, 2010).

The CheY-like fold is the most common three dimensional architecture found in the receiver domains of RRs throughout the bacterial world (Bourret, 2010). The active core of receiver domains (PFAM nomenclature: Response_rec) has a $(\beta/\alpha)_5$ topology with a central five-stranded parallel β sheet surrounded by five α helices. This structure contains several highly conserved residues. Three aspartate residues bind a divalent cation, which is essential for de-phosphorylation and phosphorylation reactions. Within this motif, the central aspartate residue (e.g. CheY-D57) is phosphorylated in the course of signal transduction (Lukat *et al.*, 1990, West & Stock, 2001). Phosphorylation of RRs by low molecular weight phospho-donors (e.g. acetyl phosphate) are described in vitro and in vivo (Wolfe, 2010), but the natural relevance still remains to be elucidated (Liu *et al.*, 2009). Phosphorylation-induced structural rearrangements of the $(\beta/\alpha)_5$ topology lead to an activation of the respective RR. In most cases, activated RRs dimerize and as a result their DNA-binding affinity is increased. In addition to the afore-mentioned mechanisms for dephosphorylation (see Chapter 1.2), the level of phosphorylated RR can also be regulated by de novo protein synthesis. Hence, many of the TCSs that regulate cellular processes on a transcriptional level are also subject to autoregulation (Groisman, 2001, Stock *et al*., 2000).

Effector domains mediate the cellular response to the perceived stimulus. In general, the sensed signal can be transduced into altered gene expression, enzymatic activity or protein-protein interactions (Galperin, 2010). Over 60 effector domain protein families have been described. Fifty-one percent of all RRs neither harbor an effector domain, nor do they have enzymatic activity (at least none has yet been characterized). DNA-binding output domains are classified according to their structure. Thirty percent of all RRs contain winged helix domains and belong to the OmpR/PhoB RR family (PFAM nomenclature: Trans_reg_c). Sixteen percent harbor helix-turn-helix domains, as found in the NarL/FixJ RR family (PFAM nomenclature: LuxR_C_like or GerE), and 3% contain LytTR domains as observed in the LytR/AgrA RR family (PFAM nomenclature: LytTR) (Finn *et al.*, 2010, Galperin, 2010, Gao & Stock, 2009). Recently, the structure of the LytTR output domain of AgrA in *Staphylococcus aureus* was elucidated (Sidote *et al.*, 2008). The structure is composed of a 10-stranded β fold of the LytTR domain, and reveals a novel mode of protein-DNA interaction.

Besides their role in controlling gene expression, RR effector domains contain enzymatic activities, like the chemotaxis-modulating methylesterases/methyltransferases CheB/CheR (Bren & Eisenbach, 2000). Others modulate the transduced stimulus via protein-protein interactions as described in the partner-switching mechanism of *Caulobacter crescentus*. Here, the anti-σ factor NepR captures and thereby inactivates the RR PhyR.

1.4 The class of LytS/LytTR-like histidine kinases/response regulators

TCSs of the LytS/LytTR HK/RR family are the second-most distributed in all bacterial genomes (Geer *et al.*, 2002). All family members consist of a LytS-like HK (with the characteristic 5TM Lyt domain) and a LytTR-like RR (with the characteristic LytTR domain). In several bacterial pathogens, virulence factors and housekeeping genes are regulated by LytS/LytTR-containing TCSs (Table 1). The eponymous LytS/LytR system of *S. aureus* (homolog to the uncharacterized LytS/LytT system in *B. subtilis*) controls autolysis of the cells (programmed cell death) and peptidoglycan turnover (Sadykov & Bayles, 2012). Quorum-sensing dependent mechanisms, such as the production of the extracellular polysaccharide, alignate, in the cystic fibrosis-associated opportunistic human pathogen, *Pseudomonas aeruginosa,* are also under the control of the LytS/LytTR-like FimS/AlgR (Lizewski *et al.*, 2004, Mohr *et al.*, 1992).

Further examples of LytS/LytTR-containing systems can be found in such common organisms as the gas gangrene-causing *Clostridium perfringens,* where VirS/VirR is implicated in the production of toxins (Shimizu *et al*., 2002, Rood, 1998), in *Streptococcus pneumonia,* where BlpH/BlpR is required for growth and for the transcriptional regulation of

Table 1. LytS/LytTR-like two-component systems

Proteins (HK/RR)	Organism	Disease	Regulated process
AgrC/AgrA	*Staphylococcus aureus*, Gram-positive bacteria	Wound infection, toxic shock syndrome	Production of exotoxins, hemolysins, staphylokinase, other secreted proteins
BlpH/BlpR	*Streptococcus pneumoniae*	Middle ear infection, pneumonia, meningitis	Production of bacteriocins (short anti-bacterial peptides), BlpR essential for growth
CabS/CabR, PlnB/PlnC	*Lactobacillus plantarum*, Gram-positive bacteria	None, many strains are probiotic (beneficial)	Production of bacteriocins (short anti-bacterial peptides)
ComC/ComE	*S. pneumoniae*, other streptococci	Middle ear infection, pneumonia, meningitis	Natural competence to DNA transformation
CoxA/CoxC	*Oligotropha carboxidovorans*, α-proteobacteria	None	Utilization of carbon monoxide, other environmental responses
FasB/FasA	*Streptococcus pyogenes*	Pharyngitis, tonsillitis, necrotizing fasciitis	Production of fibronectin-binding adhesin, streptokinase, streptolysin S
FimS/AlgR	*Pseudomonas aeruginosa*	Cystic fibrosis	Biosynthesis of extracellular polysaccharide alginate; twitching motility
FsrC/FsrA	*Enterococcus faecalis*	Endocarditis and bacteremia, urinary tract infections, meningitis	Production of virulence-related proteases, gelatinase and serine protease
HdrM/HdrR, BrsM/BrsR	*Streptococcus mutans*	Dental caries	Production of the lantibiotic mutacins I, II and III (peptide antibiotics)
LytS/LytT, LytS/LytR	*Bacillus anthracis*, *S. aureus*, Gram-positive bacteria	Anthrax, Wound infection, toxic shock syndrome	Peptidoglycan turnover, autolysis
VirS/VirR	*Clostridium perfringens, C. tetani, C. botulinum*	Gas gangrene	Production of exotoxins, collagenase, hemagglutinin

The data used to draw up this table were extracted from Galperin, 2008, Del Papa & Perego, 2011, Merritt & Qi, 2012, Dawid *et al*., 2007.

bacteriocin production (Dawid *et al.*, 2007, de Saizieu *et al.*, 2000), and in *Lactobacillus plantarum,* where PlnB/PlnC is involved in bacteriocin production (Diep *et al.*, 2003, Risøen *et al.*, 2001). The best-characterized LytS/LytTR family member is the AgrC/AgrA system of *S. aureus*, which controls a quorum-sensing system and a global virulence regulator. AgrA up-regulates genes encoding secreted virulence factors and down-regulates genes encoding cell wall-associated proteins (Sidote *et al*., 2008). Recently, as already predicted by Galperin and Nikolskaya (2002), a new type of DNA binding domain was identified in the LytTR-like RR AgrA of *S. aureus* by x-ray crystallography (Sidote *et al.*, 2008). The interaction of the RR and the target-DNA is mediated by a 10-stranded β fold of the LytTR domain (Sidote *et al.*, 2008). The sheets are arranged roughly parallel to each other in an elongated β-β-β sandwich (sheet 1: β1-β2; sheet 2: β3-5-α1-β6-7; sheet 3: β8-β10), separated by two α helices, which are not involved in DNA binding (Sidote *et al*., 2008). AgrA binds the DNA as a parallel-arranged dimer, and the consensus binding sequence consists of a 9 bp direct repeat motif separated by a 12 bp spacer (Koenig *et al.*, 2004). The LytTR binding motif (Nikolskaya & Galperin, 2002) was originally described as (T/A)(A/C)(C/A)GTTN(A/G)(T/G), but recent studies have indicated that the recognition motif of LytTR-containing RRs is more variable than previously proposed (Del Papa & Perego, 2011, Galperin, 2008).

In Gram-negative bacteria, especially the YehU/YehT and YpdA/YpdB TCS in *E. coli*, little is known about the LytS/LytTR HK/RR family (Fig. 1.2). The YehU/YehT and YpdA/YpdB TCS often co-occur in proteobacteria (Szklarczyk *et al.*, 2011). The HKs YehU and YpdA have 29% sequence identity (similarity 53%), and the RRs YehT and YpdB have 32% sequence identity (similarity 53%) (http://blast.ncbi.nlm.nih.gov/, Johnson *et al.*, 2008).

1.4.1 The two-component system YehU/YehT of *Escherichia coli*

YehU/YehT was first identified by sequence and homology analysis. It is composed of a LytS-like HK and LytR-like RR (Riley *et al.*, 2005, Mizuno, 1997). *yehU* and *yehT* form an operon with 4 bp overlap, which is localized at 47.638 centisomes in the *E. coli* MG1655 genome (Fig. 1.3 A). The operon is flanked by *mlrA* (221 bp upstream of *yehU*) and *yehS* (46 bp downstream of *yehT*) (Fig. 1.3 A). *mlrA* encodes a regulator of curli production in pathogenic *E. coli* (Brown *et al.*, 2001). The function of *yehS* is unknown. Adjacent to these genes are the genes/operons *yohO*, *osmF-yehYXW*, *yehR*, and *yehLMPQ*. *yehL* encodes a putative ATP-binding subunit of the ABC transporter family (Snider *et al.*, 2006); *yohO* encodes a small membrane protein (Hemm *et al.*, 2008); and the product of the *osmF-yehYXW* operon is a putative ABC transporter (Checroun & Gutierrez, 2004). Thus far, the functions of the products of *yehM*, *yehP*, *yehQ*, or *yehR* could not be predicted (Keseler *et al.*, 2009).

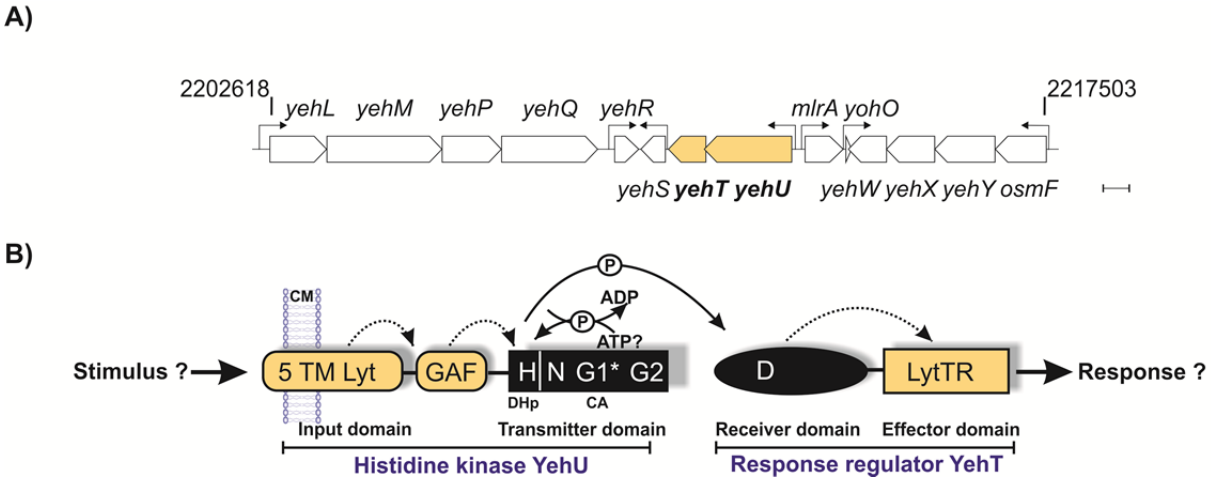

Fig. 1.3 The YehU/YehT two-component system of *Escherichia coli.* A) The region between 47.48 and 47.77 centisomes (bp 2202618 to 2217503) around the *yehUT* locus on the *E. coli* MG1655 chromosome is shown. See the text for a description of the neighboring genes. Arrows mark transcription start sites as indicated by EcoCyc (http://www.ecocyc.org, Keseler *et al.*, 2009). The bar represents 500 bp. B) Domain organization of YehU and YehT. The input domain of YehU consists of the 5TM Lyt (LytS-YhcK) domain (Anantharaman & Aravind, 2003) and a GAF domain. The G1 box of YehU is incomplete (G1*) and ATP binding can only be speculated on (ATP?). YehT is made up of a CheY-like receiver domain and a LytTR-type DNA-binding domain (Nikolskaya & Galperin, 2002). The phosphorylation sites are indicated (H, Histidine 382; D, Asparte 54). N, G1, and G2 are conserved boxes in HKs; CM, cytoplasmic membrane.

The LytS-like HK YehU consists of 561 amino acids (62.1 kDa) (Jain *et al.*, 2009, Keseler *et al.*, 2009). The N-terminal input domain of YehU consists of a 5TM Lyt (LytS-YhcK) domain (PFAM nomenclature: 5TMR-LYT) (Anantharaman & Aravind, 2003) and a GAF domain (Fig. 1.3 B). Bioinformatic analysis indicates that YehU harbors at least five membrane-spanning α-helices [according to the TMHMM, MEMSAT3, and OCTOPUS programs (Jones, 2007, Krogh *et al.*, 2001, Viklund & Elofsson, 2008)]. 5TM Lyt domains are characterized by a NXR motif in the loop between helix 1 and 2, multiple small residues, like glycine and proline, in the middle of helix 2, and a small residue (typically glycine) in the midst of the fifth helix (Anantharaman & Aravind, 2003). These small residues can distort the overall structure of TM helices and therefore might be involved in ligand binding and signal transmission (Anantharaman & Aravind, 2003). Furthermore, based on sequence homology analysis with ProDom (http://prodom.prabi.fr/, Servant *et al.*, 2002) the input domain of YehU can be divided into two subdomains. The first subdomain (amino acids 16-101; ProDom ID PD810677) consists of 85 amino acids and is conserved in pathogens like *Salmonella* or *Vibrio spp.* The second subdomain (amino acids 102-193; ProDom ID PD633472) contains the characteristic residues for the 5TM Lyt family classification (Kraxenberger, 2011). SMART, UniProt, PFAM and NCBI BLASTP databases predict histidine 382 of YehU as the crucial phosphorylation site (http://smart.embl.de, Letunic *et al.*, 2006, http://www.uniprot.org, Consortium, 2010, http://pfam.sanger.ac.uk/, Finn *et al.*, 2010, http://blast.ncbi.nlm.nih.gov/, Johnson *et al.*, 2008). Moreover, SMART, UniProt and NCBI BLASTP databases specify a GAF domain in the cytoplasmic part of the YehU input domain. Here, the exact position

differs between all databases but a core motif between amino acids 218 and amino acids 365 of YehU can be specified. GAF domains were shown to be capable of binding small molecules (e.g. binding of cGMP or ions) and/or involved in stimulus transduction (Cann, 2007). Nevertheless, in most proteins, its function remains to be elucidated (Möglich *et al.*, 2009). In YehU, the GAF domain is followed by a DHp domain and a CA domain, as illustrated in Figure 1.3 B.

The LytTR-like RR YehT consists of 289 amino acids (27.4 kDa) (Jain *et al*., 2009, Keseler *et al*., 2009) and is organized in two domains (Fig. 1.3 B). A CheY-homologous receiver domain is connected to a DNA binding domain of the LytTR family (Finn *et al*., 2010, Nikolskaya & Galperin, 2002). Based on sequence alignments, UniProt and NCBI BLASTP databases predict aspartate 54 as the phosphorylation site in YehT. Comparative secondary structure predictions and homology modeling based on the DNA-binding domain of AgrA indicated that the DNA-binding domain of YehT has a similar elongated β-β-β sandwich structural arrangement, with a reduced number of β sheets (Kraxenberger, 2011).

The specific physiologic and functional role of the YehU/YehT TCS in *E. coli* remains elusive. In *S. aureus,* the homologous LytS/LytR TCS regulates the expression of *lrgA* and *lrgB*. The homologous gene products are present in *E. coli*, but are not regulated via the YehU/YehT TCS (Kraxenberger, 2011). Various systematic studies have failed to identify either the stimulus or the target gene of the YehU/YehT TCS (Oshima *et al*., 2002, Hirakawa *et al*., 2003). Several phenotypic analyses, testing up to 2,000 different growth conditions did not show any significant phenotypic difference between the wild-type strain and an isogenic *yehUT* deletion mutant (Behr, 2009, Lorenz, 2011, Zhou *et al*., 2003). Furthermore, no significant differences with respect to cell motility, biofilm formation, cell surface hydrophobicity, curli formation, or cell morphology between *E. coli* strain MG1655 and a *yehUT* deletion mutant were observed. Moreover, in vitro characterization of all TCSs from *E. coli* identified a low phosphorylation rate for YehU/YehT and no crosstalk to other systems (Yamamoto *et al*., 2005).

1.4.2 The two-component system YpdA/YpdB of *Escherichia coli*

In the *E. coli* MG1655 genome, the genes *ypdA,* encoding for a LytS-type HK*,* and *ypdB,* encoding for a LytTR-type RR, are genetically associated with *ypdC* in the *ypdABC* operon (Fig. 1.4 A), which is located at 53.56 centisomes (Keseler *et al*., 2009). *ypdC* encodes a helix-turn-helix AraC-type regulatory protein of unknown function. The *ydpABC* operon is flanked by *alaC* (376 bp upstream of *ypdA*) and the *fryABC-ypdEF* operon (3 bp downstream of *ypdC*). AlaC is one of three major alanine-synthesizing transaminases (Kim *et al.*, 2010). The *fryABC-ypdEF* operon encodes putative components of a phosphotransferase system and two aminopeptidases (Zheng *et al.*, 2005).

The HK YpdA consists of 565 amino acids (62.7 kDa) with a N-terminal 5TM Lyt (LytS-YhcK) input domain (Anantharaman & Aravind, 2003), linked to a GAF and a transmitter domain (Fig. 1.4 B). According to bioinformatics analyses [using programs TMHMM, MEMSAT3 and OCTOPUS (Krogh *et al*., 2001, Jones, 2007, Viklund & Elofsson, 2008)], YpdA harbors at least six TM helices. As already described for YehU, the N-terminal part of the HK contains conserved residues which were predicted to be involved in ligand binding and signal transmission (Anantharaman & Aravind, 2003). Furthermore, based on sequence analysis with ProDom (http://prodom.prabi.fr/, Servant *et al*., 2002), the input domain of YpdA, as well as for YehU, can be divided into two subdomains. The first and more variable subdomain (amino acids 1-75; ProDom ID PD856107) consists of 75 amino acids and is conserved in pathogens like *Staphylococcus, Bacillus* or *Streptococcus*. The second subdomain (amino acids 86-191; ProDom ID PD633472) contains the characteristic residues for the 5TM Lyt family classification. SMART, UniProt, PFAM and NCBI BLASTP databases predict histidine 371 of YpdA as the crucial phosphorylation site (http://smart.embl.de, Letunic *et al*., 2006, http://www.uniprot.org, Consortium, 2010, http://pfam.sanger.ac.uk/, Finn *et al*., 2010, http://blast.ncbi.nlm.nih.gov/, Johnson *et al*., 2008).

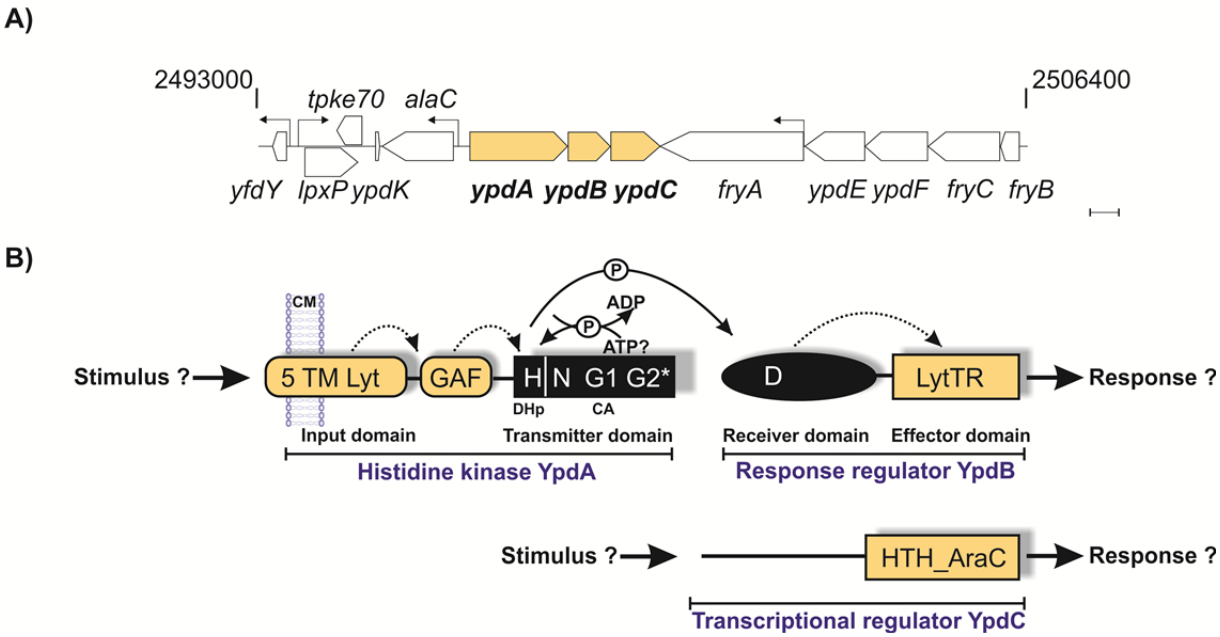

Fig. 1.4 The YpdA/YpdB two-component system of *Escherichia coli.* A) The region between 53.73 and 54.01 centisomes (bp 2493000 to 2506400) around the *ypdABC* locus on the *E. coli* MG1655 chromosome is shown. See the text for a description of the neighboring genes. Arrows mark transcription start sites as indicated by EcoCyc (http://www.ecocyc.org, Keseler *et al*., 2009). The bar represents 500 bp. B) Domain organization of YpdA, YpdB and YpdC.The input domain of YpdA consists of the 5TM Lyt (LytS-YhcK) domain (Anantharaman & Aravind, 2003) and a GAF domain. The G2 box of YehU is incomplete (G2*) and ATP binding can only be speculated on (ATP?). YpdB is made up of a CheY-like receiver domain and a LytTR-type DNA-binding domain (Nikolskaya & Galperin, 2002). The phosphorylation sites are indicated (H, Histidine 371; D, Asparte 53). N, G1 and G2 are conserved boxes in HKs. In the transcriptional regulator YpdC a helix-turn-helix motif of the AraC-type (HTH_AraC) is present; CM, cytoplasmic membrane.

Furthermore, in the cytoplasmic part of the YpdA input domain, a GAF domain is predicted. GAF domains are known to be involved in the perception of stimuli and/or signal transduction. This domain is followed by a DHp domain and a CA domain, as illustrated in Figure 1.4 B.

The LytTR-like RR YpdB consists of 244 amino acids (28.7 kDa) (Jain *et al*., 2009, Keseler *et al*., 2009) and is, like YehT, composed of a CheY-like receiver domain connected to a DNA binding domain of the LytTR family (Fig 1.4 B) (Finn *et al*., 2010, Nikolskaya & Galperin, 2002). Based on sequence alignments, UniProt and NCBI BLASTP databases predict aspartate 53 as the phosphorylation site in YpdB. Comparative secondary structure predictions and homology modeling based on the DNA-binding domain of AgrA indicate that the DNA-binding domain of YpdB has a similar elongated β-β-β sandwich structural arrangement, with a reduced number of β sheets (Kraxenberger 2011, unpublished data).

In addition to the prototypical TCS proteins, an AraC-like regulatory protein, YpdC, is present. Therefore, the system can be classified as YpdA/YpdB/YpdC three-component system. The putative transcriptional regulator YpdC consists of 285 amino acids (32.4 kDa) (Keseler *et al*., 2009) and is characterized by a putative N-terminal substrate binding domain and a C-terminal helix-turn-helix AraC-like DNA-binding domain (PFAM nomenclature: HTH_18) (Fig. 1.4 B) (Letunic *et al*., 2006). In *E. coli* AraC perceives L-arabinose as stimulus and regulates expression of genes involved in arabinose catabolism and transport (Schleif, 2010). The crystal structure of the C-terminal domain shows two helix-turn-helix DNA-binding domains connected by an α-helix (Rodgers & Schleif, 2009).
The specific physiologic and functional role of the YpdA/YpdB TCS in *E. coli* remains elusive. Various systematic studies have neither identified the stimulus nor the target gene of the YpdA/YpdB TCS (Oshima *et al*., 2002, Hirakawa *et al*., 2003, Inoue *et al.*, 2007). Furthermore, phenotypic analysis, as described in 1.4.1, did not reveal differences between wild-type and a *ypdAB* deletion mutant (Zhou *et al*., 2003, Lorenz, 2011). Functional in vitro characterization of all TCSs from *E. coli* remained elusive for the YpdA/YpdB system, as YpdA was not produced (Yamamoto *et al*., 2005). Therefore, no statement on (auto-)kinase activity of YpdA and phosphotransferase activity to YpdB can yet be issued.

1.5 Scope of this thesis

LytS/LytTR-like two-component systems are essential regulators in pathogenic Gram-positive bacteria. So far, this class of two-component systems is poorly characterized in Gram-negative bacteria. The main objective of this thesis is to elucidate the molecular mechanisms of signal perception, signal transduction, and cellular signal integration of the

LytS/LytTR-like YehU/YehT and YpdA/YpdB two-component systems in *Escherichia coli*. Further aims are the development of new approaches to analyze transcriptional and translational regulatory processes.

In order to address these tasks in the first study of this work, it will be necessary to develop convenient tools to facilitate the rapid construction of reporter *lacZ* fusions, which could then be used to generate chromosomal- and plasmid-encoded fusions.

With the right tools in hand, a comprehensive characterization of the YehU/YehT system will be carried out in the second study. Target genes shall be identified and further characterized.

The third study will attempt to shed light on how the YpdA/YpdB system creates a distinct cellular response. Using transcriptional and biochemical analysis, YpdB- and stimulus-dependent expression of target genes shall be demonstrated.

Since the second and third studies focus on system-specific responses, the fourth study shall demonstrate that the YehU/YehT and YpdA/YpdB systems cooperate in a network-like fashion. To achieve this, a combination of expression analysis and protein-protein interaction studies between the system components and accessory proteins, shall be employed.

Finally, the fifth study shall address the question of how accessory proteins are integrated to mediate signal integration, scaffolding, interconnection and allosteric regulation, and how these “two-components” are embedded in regulatory networks.

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2 *lacZ* reporter strategies

2.1 A comprehensive toolbox for the rapid construction of *lacZ* fusion reporters

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Running title: *lacZ* reporter strategies

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Abstract

β-Galactosidase encoded by *lacZ* remains a popular reporter enzyme. Here, we present three fast and convenient tools that facilitate rapid construction of reporter *lacZ* fusions. The first enables the simple generation of *lacZ* (*slacZ*)-based chromosomally encoded reporter fusions within the *lac* operon in *Escherichia coli* using Red®/ET® recombination. The *slacZ* tool is based on *rpsL* counter-selection in combination with homologous recombination catalyzed by the λ Red recombinase, and blue/white screening. This permits construction of transcriptional and translational reporter *lacZ* fusions within a day. The second tool allows the introduction of *lacZ* reporter fusions into the chromosome by a single-crossover method. The strategy relies on the γ-origin-based suicide vector pNPTS138-R6KT, which can only replicate in λ*pir E. coli* strains. The third tool comprises four pBBR1-based broad-host-range vectors for transcriptional and translational *lacZ* fusions. The functionality of our toolbox was confirmed by the K^+-dependent activation of *kdp* promoter-*lacZ* fusions *in vivo*.

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2.2 Repoter gene fusions for LytS/LytTR-like signaling systems

The target genes of the LytS/LytTR-like signaling systems YehU/YehT and YpdA/YpdB in *E. coli* are *yjiY* and *yhjX*, respectively (see chapters 3 and 4). So, the *lacZ* tools (chapter 2.1) were used to analyze *yjiY* and *yhjX* expression. Chromosomal promoter *lacZ* fusions were constructed resulting in *E. coli* LF4 (P_{yjiY}*::lacZ)* and *E. coli* LF5 (P_{yhjX}*::lacZ)*. Cells were grown in LB medium under agitation and the activation of the corresponding promoters were tested using β-galactosidase activity assays (Fig. 2.2). Here, even under artificial activation of the systems (overproduction of the corresponding RR), the monitored expression levels of

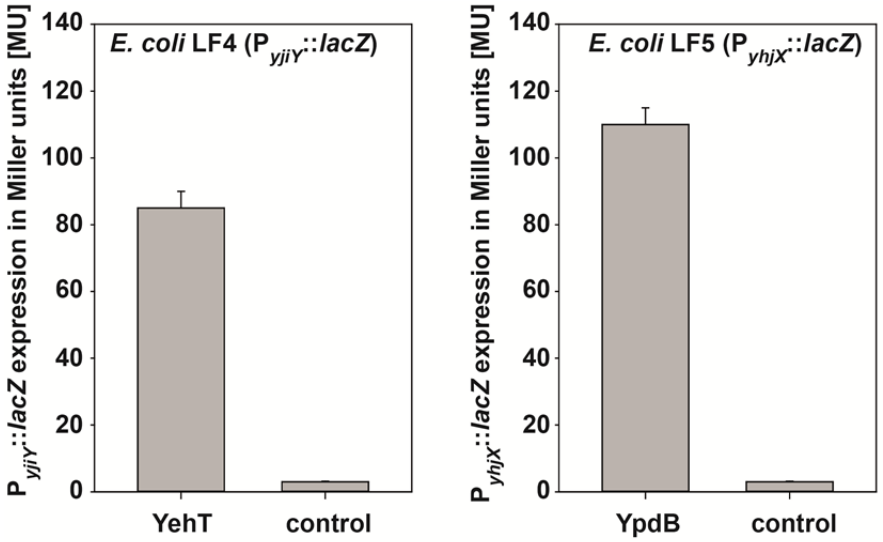

Fig. 2.2 Artificial stimulation of *yjiY* or *yhjX* expression. *E. coli* LF4 *(P_{yjiY}::lacZ) and E. coli* LF5 *(P_{yhjX}::lacZ)* were transformed with either plasmid pBAD24 (control) or a plasmid encoding the corresponding YehT (LF4: pBAD24-*yehT*) or YpdB (LF5: pBAD24-*ypdB*). Bacteria were cultivated under aerobic growth conditions in LB medium at 37°C until the exponential growth phase. Then overexpression was induced by adding 0.2% (wt/vol) L-arabinose, and cells were harvested after 45 min. β-galactosidase (LacZ) activity was then determined as a measure of *yjiY* or *yhjX* promoter activity. Experiments were performed at least three times, and error bars indicate the standard deviations of the means.

chromosomal promoter activity were low (Fig. 2.2). So, new plasmid-based strategies to analyze the target gene expression in more detail were elaborated: 1. A transcriptional fusion of the *yjiY* (P_{yjiY}) and *yhjX* (P_{yhjX}) promoter and the luciferase operon *luxCDABE* in the pBBR1-backbone were generated (see chapters 3 and 4). These tools offer a suitable alternative as they allow in-growth detection of expression, show a significantly higher sensitivity and are of these reasons an advantageous reporter system for the LytS/LytTR-like HKs/RRs. 2. Identical promoter fusions to *lacZ* in the high copy number vector pRS415 were also used to characterize the RR binding sites (see chapters 3 and 4).

Nevertheless, the introduced *lacZ* toolbox is a beneficial system as it allows any chromosomal and plasmid-encouded *lacZ* fusion in a broad-host-range of organisms. Beside the limitations for the LytS/LytTR-like TCSs this toolbox is used in several molecular applications (Ude, unpublished data; Lassak, unpublished data; Müller, unpublished data).

3 First insights into the unexplored two-component system YehU/YehT in *Escherichia coli*

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Running title: Response regulator YehT in *E. coli*

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Abstract

Two-component systems (TCSs) consisting of a membrane-anchored histidine kinase (HK) and a response regulator (RR) are major players in signal transduction in prokaryotes. Whereas most TCSs in *Escherichia coli* are well characterized, almost nothing is known about the LytS-like HK YehU and the corresponding LytTR-like RR YehT. To identify YehT-regulated genes, we compared the transcriptomes of *E. coli* cells overproducing either YehT or the RR KdpE (control). The expression levels of 32 genes varied by more than 8-fold between the two strains. A comprehensive evaluation of these genes identified *yjiY* as a target of YehT. Electrophoretic mobility shift assays with purified YehT confirmed that YehT interacts directly with the *yjiY* promoter. Specifically, YehT binds to two direct repeats of the motif ACC[G/A]CT[C/T]A separated by a 13-bp spacer in the *yjiY* promoter. The target gene *yjiY* encodes an inner membrane protein belonging to the CstA superfamily of transporters. In *E. coli* cells growing in media containing peptides or amino acids as carbon source, *yjiY* is strongly induced at the onset of the stationary growth phase. Moreover, expression was found to be dependent on cAMP/CRP. It is suggested that YehU/YehT participates in the stationary phase control network.

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4 Identification of a target gene and activating stimulus for the YpdA/YpdB histidine kinase/response regulator system in *Escherichia coli*

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Abstract

Escherichia coli contains 30 two-component systems (TCSs), each consisting of a histidine kinase and a response regulator. Whereas most TCSs are well characterized in this model organism, little is known about the YpdA/YpdB system. To identify YpdB-regulated genes, we compared the transcriptomes of *E. coli* cells overproducing either YpdB or a control protein. Expression levels of 15 genes differed by more than 1.9-fold between the two strains. A comprehensive evaluation of these genes identified *yhjX* as sole target of YpdB. Electrophoretic mobility shift assays with purified YpdB confirmed its interaction with the *yhjX* promoter. Specifically, YpdB binds to two direct repeats of the motif GGCATTTCAT separated by an 11-bp spacer in the *yhjX* promoter. *yhjX* encodes a cytoplasmic membrane protein of unknown function that belongs to the Major Facilitator Superfamily of transporters. Finally, we characterized the pattern of *yhjX* expression and identified extracellular pyruvate as a stimulus for the YpdA/YpdB system. It is suggested that YpdA/YpdB contributes to nutrient scavenging before entry into stationary phase.

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5 Identification of the LytS/LytTR-like signaling network in *Escherichia coli*

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Key words: *yhjX, yjiY,* histidine kinase, response regulator, LytTR transcriptional regulator, major facilitator superfamily of transporters, peptide transporter, protein-protein interaction

5.1 Abstract

Two-component systems (TCSs) consisting of a membrane-anchored histidine kinase (HK) and a response regulator (RR) are major regulators in signal transduction of prokaryotes. Whereas most TCSs in *Escherichia coli* are well characterized as single signaling units, almost nothing is known about the organization of HKs and RRs in networks.

Previously, we identified the target genes *yjiY,* encoding a putative peptide transporter, and *yhjX*, encoding a putative Major Facilitator Superfamily transporter, of the LytS/LytTR-like HK/RR YehU/YehT or YpdA/YpdB, respectively. Here, we report how the LytS/LytTR class of TCS in *E. coli* is embedded in a complex regulatory network: A transient expression of *yjiY* and *yhjX* was determined to be in mid-exponential growth phase. In addition, we identified *yehS*, encoding a putative accessory protein, which is induced directly after *yjiY* and *yhjX*. Moreover, an in vivo and in vitro interaction of YehS with the YehU/YehT and YpdA/YpdB signaling cascade was demonstrated. The interaction of YehS with the GAF-domain of the HKs YehU and YpdA was specified. Furthermore, YehS-YehT or YehS-YpdB interactions were influenced by the phosphorylation state of the RRs.

Concordantly, in vivo expression analysis demonstrated a physiologic connection between the two systems. Moreover, Carbon storage regulator A (CsrA) was found to post-transcriptionally regulate *yjiY* and *yhjX*. Finally, we present a model of the LytS/LytTR-like signaling network in *E. coli*.

5.2 Introduction

Bacteria have to sense and respond to changing environmental conditions in order to survive. Two-component systems (TCSs) are the major players in prokaryotic signal transduction. A membrane-bound histidine kinase (HK) senses a stimulus and transduces it into a cellular signal via phosphorylation. The transfer of this phosphoryl group to a response regulator (RR) with DNA-binding properties mediates the reaction, generally an alteration in gene expression (Jung *et al.*, 2012).

In *Escherichia coli* 30 HKs and 32 RRs have been annotated (Heermann & Jung, 2010) and most of them are target of intense studies. Recently, the YehU/YehT-system and the YpdA/YpdB-system were characterized (Kraxenberger *et al.*, 2012, Fried *et al.*, 2012). Both systems belong to the LytS/LytTR-class of TCSs with a LytS-like HK and a LytTR-like RR, are widely distributed among proteobacteria, and share the same structural domain assembly including an amino acid identity of over 30% (Riley *et al.*, 2005, Anantharaman & Aravind, 2003, Szklarczyk *et al.*, 2011). LytS/LytTR family members have often been described to regulate crucial host-specific mechanisms in human- and plant pathogenic bacteria (Galperin, 2008). Bioinformatical analyses of the HKs (using programs TMHMM,

MEMSAT3 and OCTOPUS (Krogh *et al.*, 2001, Jones, 2007, Viklund & Elofsson, 2008) propose at least five transmembrane helices for the input domains of the 5TM Lyt (LytS-YhcK) type (Anantharaman & Aravind, 2003). In addition, *E. coli's* HKs YehU and YpdA harbor a GAF domain, which is commonly found in cyclic GMP (cGMP)-specific phosphodiesterases, adenylyl cyclases, and the FhlA protein (hence GAF) and is capable, e.g., of binding cGMP, proteins, and ions (Cann, 2007, Zoraghi *et al.*, 2004), but its function in most proteins is still unknown (Möglich *et al.*, 2009).

The RRs YehT and YpdB comprise of a N-terminal CheY-like receiver domain and C-terminal LytTR-like effector domain with DNA-binding affinity (Nikolskaya & Galperin, 2002). Under activating conditions YehT induces *yjiY*, encoding for a putative peptide transporter (Kraxenberger *et al*., 2012). *yhjX*, encoding for an uncharacterized MFS transporter, is induced by YpdB (Fried *et al*., 2012). The YehU/YehT and YpdA/YpdB system have stimulating conditions in common (e.g. LB-medium), here expression takes places in mid-exponential growth phase. Both systems have also unique inducers.

The discovery of accessory proteins influencing the TCS signaling is an emerging field in bacterial signaling. Several mechanisms how accessory proteins modulate TCS signaling has been described (Jung *et al*., 2012, Buelow & Raivio, 2010): Co-sensing, signal integration, scaffolding, interconnection and allosteric regulation accomplished by accessory proteins are some so far described examples. These accessory regulators are widespread and are localized in all bacterial compartments. Interactions of them with sensing, transmembrane, signal integration, enzymatic or DNA binding domains in HKs and RRs have been identified. The activity of accessory proteins is controlled by differential expression, modification and/or ligand binding. Furthermore, accessory proteins can connect TCS to other pathways/networks to broaden the range of sensed stimuli and/or to build distinct hubs to control the cellular flow of information.

In this study we identified and characterized the LytS/LytTR-TCS network in *E. coli*. We described coordinated expression of *yjiY*, *yhjX* and *yehS*. Furthermore, we demonstrated that the accessory protein YehS interacts in vivo and in vitro with all TCS components. Moreover, our results indicate the participation of this network in the carbon control.

5.3 Materials and Methods

Strains, plasmids and oligonucleotides. *E. coli* strains and their genotypes are listed in Table 5.1. Mutants were constructed by using the *E. coli* Quick and Easy Gene Deletion Kit (Gene Bridges) and the Bac Modification Kit (Gene Bridges) as reported (Heermann *et al.*, 2008). Both kits rely on the Red/ET recombination technique. Plasmids (≥100) and all oligonucleotides (≥400) used in this work are available on request. DNA fragments for construction were amplified by PCR from genomic DNA.

Table 5.1 Bacterial strains used in this study

E. coli strains	Relevant genotype or description	Reference or source
MG1655	F⁻ λ⁻ *ilvG rfb50 rph-1*	(Blattner *et al.*, 1997)
MG2	MG1655 Δ*yehUT*	(Kraxenberger et al., 2012)
MG20	MG1655 Δ*ypdABC*	(Fried et al., 2012)
MG30	MG1655 Δ*yehUT* Δ*ypdABC*	This work
MG31	MG1655 Δ*csrA*	This work
MG1655-Δ*lacZ*	MG1655 Δ*lacZ*::Tet^r	K. Jahreis (personal gift)
BL21(DE3)	F⁻ *ompT hsdSB*(r^-_B, m^-_B) *gal dcm* (DE3)	(Studier & Moffatt, 1986)
BTH101	F⁻ *cyaA*-99 *araD*139 *galE*15 *galK*16 *rpsL*1 *hsdR* ^{2} µ*rA1* µ*rB1*	(Karimova *et al.*, 1998)
DH5α	F⁻ λ⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+)	(Meselson & Yuan, 1968)

Molecular biological techniques. Plasmid DNA and genomic DNA were isolated using the HiYield Plasmid Mini-Kit (Suedlaborbedarf) and the DNeasy Blood and Tissue Kit (Qiagen), respectively. DNA fragments were purified from agarose gels using the Hi-Yield PCR Clean-up & Gel Extraction Kit (Suedlaborbedarf). Phusion High-Fidelity DNA polymerase or Phire Hot Start DNA polymerase (Finnzymes) were used according to the supplier's instructions. Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs and used according to the manufacturer's directions.

Growth conditions. *E. coli* MG1655 strains (Table 5.1) were grown overnight in lysogeny broth (LB) or M9 minimal medium with 0.4 % (w/v) Glucose as C source. After inoculation bacteria were routinely grown in LB medium or M9 minimal medium with indicated C sources under agitation (200 rpm) at the designated temperature. For solid media, 1.5% (w/v) agar was added. Where appropriate, media were supplemented with antibiotics (ampicillin sodium salt: 100 µg/ml; chloramphenicol: 35 µg/ml; kanamycin sulfate: 50 µg/ml; tetracycline: 12.5 µg/ml; streptomycin: 50 µg/ml gentamycin sulfate: 50 µg/ml).

RNA isolation, cDNA synthesis and qRT-PCR. At indicated time points cells were harvested, total RNA was isolated essentially as described previously (Aiba *et al.*, 1981) and treated with DNase I for 30 min to remove residual chromosomal DNA. Subsequently, RNA was purified using the RNA Pure Kit (Suedlaborbedarf). The RNA was then used as the template for random-primed first-strand cDNA synthesis according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) (iQ5 real-time PCR detection system,

Biorad) was performed using the synthesized cDNA, a SYBR-green detection system (Biorad) and specific internal primers for *yehU*, *yehT*, *yjiY*, *yehS*, *ypdA*, *ypdB*, *ypdC, yhjX* and *recA*. Duplicate samples from three independent biological experiments were used, and the CT value (cycle threshold) was determined after 40 cycles using the iQ software (Biorad). Values were normalized with reference to *recA* and relative changes in transcript levels were calculated using the comparative C_T method (Schmittgen & Livak, 2008).

Purification and Phosphorylation of 6His- and Strep-tagged Proteins. Purification of the 6His-tagged RRs and 6His-YehS was performed as described earlier (Kraxenberger *et al*., 2012). Purification of Strep-YehS was based on overproduction of pASK IBA13+ *yehS* in *E. coli* BL21(DE3) and performed according to manufacturer's directions (IBA). Proteins were about 95% pure as judged by SDS-PAGE (Laemmli, 1970) and Western blotting using the anti-His-Tag or a HRP-Strep-Tactin antibody.

β-Galactosidase activity assay for BACTH system. *E. coli* BTH101 was transformed with pUT18 or pUT18C and pKT25 or pKT25N derivatives harboring the interaction partners of interest. Cultures were grown microaerobically overnight at 30°C in LB medium supplemented with 1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG). Cells were harvested and ß-galactosidase activities were measured as described previously (Tetsch *et al.*, 2008). Values are given in Miller units calculated according to Miller (Miller, 1992).

Surface plasmon resonance (SPR) spectroscopy. SPR measurements were performed with a Biacore™ T200. To test the interaction of Strep-YehS with the RRs, we used the method of amine-coupling for StrepMAB-Immo antibody on carboxymethyl dextran sensor chips (CM5) according to the manufacturer's instructions. Briefly, flow cells were activated by injecting a 1:1 mixture of N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Flow cell (FC) 2 was loaded with approximately 5 µg of StrepMAB-Immo antibody generating ~12,000 resonance units (RUs) before FC1 and free binding sites on FC2 were saturated with 1 M ethanolamine/HCl pH 8.0. Preparation of the chip surface was done at a flow rate of 10 µl/min. Based on our protein purification protocols, we established an optimized running buffer (50 mM Tris/HCl pH 7.6, 150 mM NaCl, 10% glycerol, 3 mM Ethylenediaminetetraacetic acid (EDTA), 0.05% Tween20®) to test interactions between Strep-YehS and the 6His-RR proteins. All experiments were done with a flow rate of 20 µl/min and at 25°C. Binding of 0.3 µM Strep-YehS was limited to 60 seconds generating an average of ~120 RUs. Rising concentrations (0.1 to 5 µM) of the 6His-RR were analyzed with a contact time of 120 seconds up to complete dissociation after 1,200 seconds.

In the second approach we examined protein-protein interactions upon RR-DNA binding. Therefore 5'-biotinylated primers,

5'-biotin-GGGGCCTTTGCCGCTCAACCGCAAAACTGACCGCTTACATCCCTAAAATAACCACTCAGTTAGGGG-3'
5'-biotin-GGGGCGCGTCATTCATTCCTGAACTAAGGCATTTCATTCCGTTCTGATGGCATTTCATGCCGGGGG-3'

comprising the YehT- and YpdB-binding sites, were mixed with equal amounts of their complementary primers and heated to 95°C for 5 minutes. After annealing at room temperature the received double-stranded DNA was captured on a streptavidin-coated CM5 chip (SA chip) to approximately ~100 RU using the running buffer (see above). The reference cell remained untreated. To avoid multimerization 500 nM of purified 6His-RR was injected for 60 seconds and additionally washed with running buffer for 60 seconds. Injection of 6His-YehS (0.01 µM to 5 µM) was carried out for 120 seconds and dissociation was monitored for 600 seconds. Regeneration of SA chip surface was achieved by injecting 1M NaCl for 10 seconds. Sensorgrams were recorded using the Biacore T200 control software and analyzed with the Biacore T200 evaluation software. The surface of flow cell 1 was used to obtain blank sensorgrams for subtraction of bulk refractive index background. The referenced sensorgrams were normalized to a baseline of zero.

In vivo expression studies. In vivo expression of *yhjX* and *yjiY* was probed with luciferase-based reporter gene assays using the pBBR-*yjiY-lux,* pBBR-*yhjX-lux,* pBBR-*yjiY'-'lux,* or pBBR'-'*yhjX-lux* plasmids in *E. coli* MG1655, respectively (Table 5.1).

Cells of an overnight culture grown in M9 minimal medium with 0.5% (w/v) glucose as C-source were inoculated into LB medium or M9 minimal medium (supplemented with different C-sources [20 mM or 0.4 %]) resulting in an OD_{600} of 0.05. Cells were grown under aerobic growth conditions at 37°C, and OD_{600} and luminescence were measured continuously. Optical density of cultures was determined in a microplate reader (Tecan Sunrise) at 600 nm. Luminescence levels were determined in a Centro LB960 (Berthold Technology) for 0.1 s, and are reported as relative light units [counts s^{-1}] (RLU).

5.4 Results and Discussion

Coordinated expression of *yhjX, yjiY* and *yehS* in mid-exponential growth phase. Previously**,** the expression of *yjiY* and *yhjX* was determined when the population was in mid-exponential growth phase. These data were based on indirect promoter luciferase activity assays under inducing conditions (Kraxenberger *et al*., 2012, Fried *et al*., 2012). Therefore, we got interested in the direct mRNA expression profiles of the genes encoding for the TCS components (*yehUT, ypdABC*), the corresponding target genes (*yjiY, yhjX*) and a protein of unknown function (*yehS*). We analyzed the transcript levels of these genes in *E. coli* strain MG1655 at different time points during growth in LB medium (shifted from non-inducing minimal medium with glucose as C-source), which is characterized by an induction for both TCSs. Cells were cultivated, RNA was isolated, cDNA synthesized and level of transcripts were determined by qRT-PCR (Fig. 5.1). Changes in mRNA levels relative to the *recA* transcript were calculated using the C_T method (Schmittgen & Livak, 2008). *yjiY* and *yhjX* expression started at an optical density (OD_{600}) of 0.4 and increased to a maximum at OD_{600}

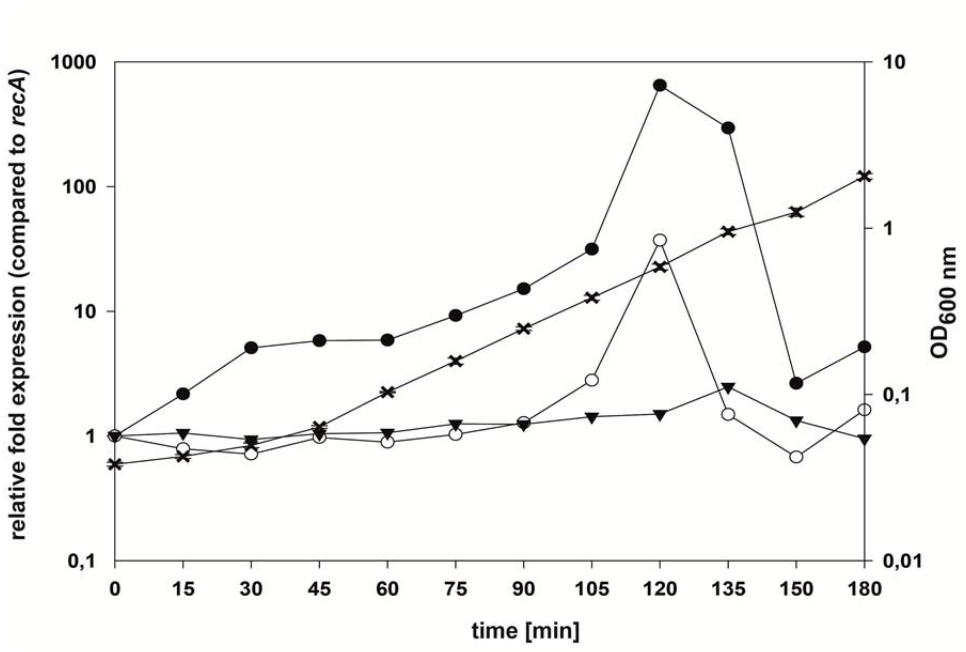

Fig. 5.1 Transcriptional analysis of the LytS/LytTR-like target genes *yhjX*, *yjiY* and the associated gene *yehS*. Cells of the wild-type (MG1655) were shifted from a stationary phase culture of M9 medium with glucose as C-soure in LB medium and grown as described in Material & Methods. Total RNA was isolated at different time points (marked by the crosses) in all growth phases and cDNA synthesized. Levels of *yjiY* (●), *yhjX* (○), *yehS* (▼) and *recA* (as reference) transcripts were determined by qRT-PCR for each time point. Changes in transcript levels (expressed relative to *recA*) were calculated using the C_T method. Relative transcript levels were normalized to 0 min values. All experiments were performed in triplicate and mean values are shown, the standard deviations were below 15 %.

~ 0.6 (*yjiY* 118 fold, *yhjX* 37 fold). Afterwards the mRNA levels decreased and when the cell density was above OD_{600} ~ 1.2 no induction was determined (Fig. 5.1). We analyzed mRNA levels till the late stationary phase but determined no additional induction (data not shown). Transcript levels were also analyzed for *yehS*. The profile for *yehS* demonstrated a growth phase independent basal expression as well as a growth phase dependent induction (2.3 fold). Interestingly, the additional induction of *yehS* started right after *yjiY* and *yhjX* and returned to basal levels when no target gene expression was detectable (Fig. 5.1, Fig. S1). The level of *yehU*, *yehT*, *ypdA*, *ypdB* and *ypdC* mRNA were constantly expressed on a low level and an additional induction was detectable when the target genes were expressed (Fig. S1). This suggets that an additional regulatory protein is influencing expression of TCS components. Such a scenario is well known for many regulatory system as the global regulator HNS binds the promoter region of the acdic stress response Cad system in *E. coli* and thereby affects expression (Krin *et al.*, 2010). Further studies will focus on the identification of this additional regulatory protein. In summary, a coordinated expression of *yjiY*, *yhjX* and *yehS* was detectable in mid-exponential growth phase.

Identification of protein-protein interactions between the LytS/LytTR regulatory network of *E. coli*. Expression analysis demonstrated a coordinated expression of the genes *yjiY*, *yhjX* and *yehS* and raised the possibility of functional connectivity between the encoding proteins. To identify possible points of contact, all genes, which have been found to influence *yjiY* or *yhjX* expression, were cloned into the BACTH vectors and analyzed for interactions. Taking into account that adenylate cyclase-based fusion proteins might be functionally restricted we probed every interaction pair in all possible N- and C-terminal combinations of the CyaA-T18 and CyaA-T25 fragments. Interactions shown are representatives for the mean value of all tested combinations. An overview is given in Table 5.2.

Table 5.2 Summary of all BACTH tested protein-protein interaction pairs.

Protein A	Protein B	A-T18 + T25-B	A-T18 + B-T25	T18-A + T25-B	T18-A + B-T25	T25-A + B-T18	T25-A + T18-B	A-T25 + B-T18	A-T25 + T18-B
Interactions of systems components									
YehU	YehU	+	-	+	+	n.d.	n.d.	n.d.	n.d.
YehU	YehT	-	+	+	+	+	-	-	+
YehU	YehS	+	-	+	-	+	+	-	+
YehT	YehT	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YehT	YehS	+	-	+	-	-	+	-	+
YehT D54E	YehT D54E	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YehT D54E	YehS	+	-	+	-	-	+	-	+
YpdA	YpdA	-	-	-	-	n.d.	n.d.	n.d.	n.d.
YpdA	YpdB	+	+	+	+	+	+	+	+
YpdA	YehS	+	-	-	-	-	+	+	+
YpdB	YpdB	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YpdB	YehS	+	-	+	+	-	+	+	+
YpdB D53E	YpdB D53E	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YpdB D53E	YehS	+	-	+	+	+	-	-	+
YehS	YehS	-	-	-	-	n.d.	n.d.	n.d.	n.d.
Interactions of target gene products									
YehU	YjiY	+	+	-	+	-	-	-	+
YehU	YhjX	-	+	+	+	-	-	+	+
YpdA	YjiY	+	-	-	+	+	+	+	+
YpdA	YhjX	+	+	+	+	+	+	+	-
YhjX	YhjX	+	-	+	-	n.d.	n.d.	n.d.	n.d.
YjiY	YjiY	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YhjX	YjiY	+	+	-	+	+	+	+	-
YjiY	YehS	-	-	-	-	-	-	-	-
YhjX	YehS	-	-	-	-	-	-	-	-
Potential crosstalk interactions									
YehU	YpdA	-	-	-	-	-	-	-	-
YehU	YpdB	-	-	-	-	-	-	-	-
YpdA	YehT	-	-	-	-	-	-	-	-
YpdB	YehT	-	-	-	-	-	-	-	-
GAF domain interactions									
YehU_GAF	YehU_GAF	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YpdA_GAF	YpdA_GAF	+	+	+	+	n.d.	n.d.	n.d.	n.d.
YehU_GAF	YehS	-	+	+	+	+	+	-	+
YpdA_GAF	YehS	-	-	-	-	-	+	-	+
Negative controls									
YehU	LysP	-	-	-	-	-	-	-	-
YpdA	LysP	-	-	-	-	-	-	-	-
YjiY	LysP	-	-	-	-	-	-	-	-

symbols: +, positive interaction; -, no interaction; n.d., not determined. Isolated GAF domains are indicated (_GAF).

With this screen we found first evidence for the dimerization of the HK YehU (Table 5.2) and an interaction between YehU and its cognate RR YehT (Fig. 5.2A). In addition an interaction between YehU and YehS was observed (Fig. 5.2A). It is suggested, that this interaction is mediated via the GAF-domain of YehU, as it was not observed in YehU ΔGAF mutant, whereas the isolated GAF-domain fusion was again found to interact with YehS (Table 5.2).

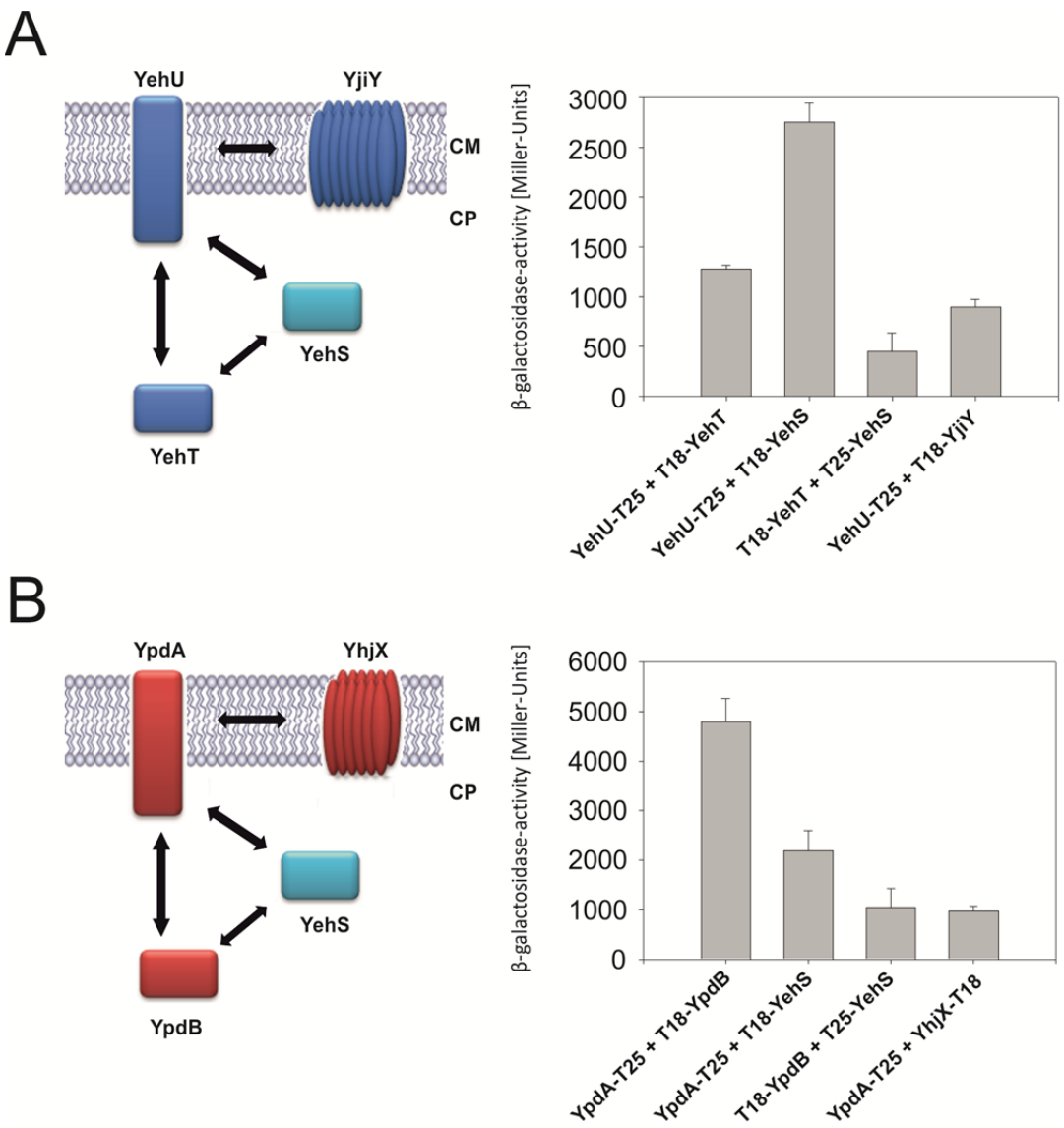

Fig. 5.2 YehS-mediated protein-protein interactions between the TCSs YehU/YehT and YpdA/YpdB and their target gene products. The BACTH system is based on restoring the adenylate cyclase (AC) activity via possible interactions of different protein-AC fusions resulting in an increased cAMP level, which can be monitored in the expression of e.g. *lacZ*, for β-galactosidase activity. To quantify the interactions, cells were cultivated aerobically in LB medium at 30°C overnight. A) All identified interactions of the YehU/YehT TCS are depicted schematically. β-galactosidase activities for selected interaction pairs are given as representatives. B) The YpdA/YpdB interactions were described and quantified according to the same principle. CM, cytoplasmic membrane; CP, cytoplasm.

Interestingly, we detected only a weak interaction of YehS and wild-type YehT, which was found to be much stronger, if the phosphorylation independent variant YehT-D54E was tested. This phenomenon was also observed for dimerization of the RR (Table 5.2).

Also using the BACTH system earlier studies validated this method to be particularly appropriate for membrane proteins (Karimova *et al.*, 2005). Known from literature many signal transduction systems integrate regulatory aspects via protein-protein interactions within the membrane (Tetsch *et al*., 2008, Kleefeld *et al.*, 2009). To Figure out, if YjiY, a putative peptide transporter under the direct control of the YehU/YehT TCS, influences its own regulation, we screened for interactions and found first evidence for a coordinated interplay of the HK YehU and its target gene product YjiY (Fig. 5.2A).

According to the same principle, we analyzed the YpdA/YpdB TCS. Although we found no proof of dimerization for the HK YpdA, the BACTH system revealed a strong interaction between the HK YpdA and the RR YpdB (Fig. 5.2B). Further an interaction between YpdA (GAF-domain of YpdA) and YehS was identified as well as an interaction between wild-type YpdB (and the phosphorylation-independent YpdB-D53E variant, respectively) and YehS (Fig. 5.2B). Also RR dimerization for wild-type YpdB as well as YpdB-D53E was observed (Table 5.2). We also identified an interaction for the HK YpdA and its target gene product YhjX, a putative MFS transporter (Fig. 5.2B).

No interactions were found for YpdA/YehU or YpdB/YehT heterodimerization and also SK/RR crosstalk, regarding interactions of YehU/YpdB and YpdA/YehT, was not detectable. Taking into account, that YpdA/YpdB and YehU/YehT share an overall amino acid identity of >30% (GAF domain YpdA/YehU: 31.7%, receiver-domain YpdB/YehT 40.7%) it is not surprising that YehS is able to interact with all four components. Although the regulatory function of GAF domains is commonly described as small ligand-binding domains in phosphodiesterases, GAF domains were also reported as contact sites for protein-protein interactions (Zoraghi *et al*., 2004). In comparison to interactions of *Pseudomonas fluorescens* GacS/GacA TCS compounds (Workentine *et al.*, 2009), it is worth mentioning, that all tested protein-protein interactions for *E .coli* LytS/LytTR TCSs revealed 5- to 10-fold higher levels of β-galactosidase enzyme activity. This could point out, that protein-protein interactions, might play a superior role in coordination of *E. coli* LytS/LytTR signal transduction, as it was shown for e.g. *Bacillus subtilis* TCS YycF/YycG and its membrane-bound regulators YycH and YycI (Szurmant *et al.*, 2007).

Screening for further interactions within this network, we found in addition an amazing connection: whereas the HKs YehU/YpdA were shown to interact with their corresponding target gene products (see above), we also identified interactions between YehU and YhjX, as well as interactions between YpdA and YjiY (Table 5.2). This was even more exceptional as we found further hints for homo- and heterodimization of YjiY/YhjX (Table 5.2).

With respect to environmental conditions it was shown, that the fumarate responsive HK DcuS of *E. coli* is also able to interact with two transport proteins DctA and DcuB (Witan *et al.*, 2012).

Due to the artificial approach of overproduction, it is likely, that the interaction of HKs (YehU and YpdA) and transport proteins (YjiY and YhjX) is enhanced and therefore independent of environmental stimuli. Nevertheless, the specificity remains remarkable, while we were not able to identify any interaction for the HKs or transport proteins with negative control (LysP, a lysine permease) (Table 5.2).

Validation of the cytosplasmic interactions between YehS and the RRs via SPR-measurements

To validate the identified interactions between the soluble compounds of the LytS/LytTR regulatory network in vitro, we purified the proteins 6His-YehT, 6-His-YehT-D54E, 6His-YpdB, 6His-YpdB-D53E, 6His-YehS and YehS-Strep, respectively. Consequently, for the verification of the BACTH analysis two different approaches were chosen: In the first experiment we immobilized Strep-tagged YehS via antibodies captured on a Biacore CM5 sensor chip and subsequently probed increasing amounts of 6His-YehT (Fig. 5.3A) and 6His-YpdB (Fig. 5.3B) and their derivatives (data not shown). Showing no dimerization in the BACTH screen (Table 5.2) we tested in addition 6His-YehS (Fig. 5.3C) as negative control.

To achieve equal starting conditions the chip surface was completely regenerated with each cycle and continually loaded with comparable amounts of Strep-YehS (time point -100 seconds). Traces given (0.25 and 2.5 µM) represented various analyte concentrations in a range from 0.1 to 5 µM. The analyte was injected (time point 0) for 120 seconds. Subsequent dissociation was monitored for 20 minutes.

The SPR binding curves showed specific binding of 6His-YehT (Fig. 5.3A) and 6His-YpdB (Fig. 5.3B) to the immobilized analyte (=Strep-YehS), whereas no interaction was observed for 6His-YehS (Fig. 5.3C). Higher relative RUs with increasing RR concentrations support the specificity of the interaction. However, the capacity for interactions was highly exceeded for RR concentrations >0.75 µM and showed no satiating effect, indicating an additional interaction event polluting an accurate measurement.

Prior studies focusing on members of the LytTR-like protein family generally struggled with the high tendency of these proteins to form multimeric complexes (Galperin, 2008, Kraxenberger *et al.*, 2012). Taking into account, that RR dimerization is a common feature in gene regulation (Gao & Stock, 2009, Capra & Laub, 2012) and was furthermore observed with the BACTH screen (Table 5.2), an additional di- or multimerization of RR molecules could explain the observed SPR data. Taking all aspects into consideration the given K_D values of about 85 nM result from at least three different experiments limited to RR

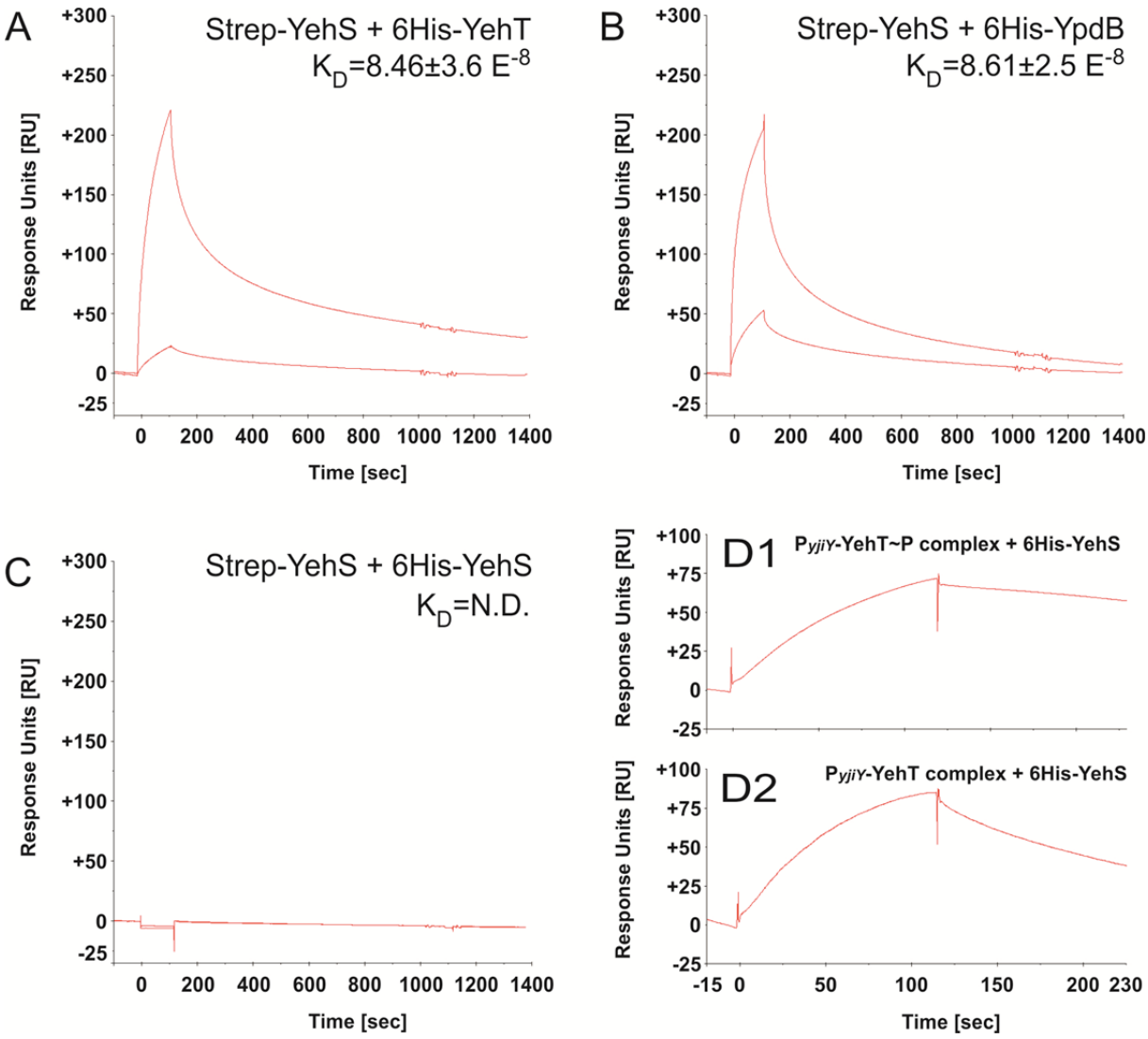

Fig. 5.3 Biochemical characterization of the interaction between YehS and the RRs YehT and YpdB using SPR spectroscopy. Strep-tagged YehS was purified and coupled via Strep-MAB antibodies to the surface of a CM5 sensor chip. The sensor chip was immobilized with constant surface densities of about 20 response units (RU). After immobilization of Strep-tagged YehS, (A) purified 6His-YehT, (B) 6His-YpdB and (C) 6His-YehS (as a negative control) was injected in a concentration range from 0.1 to 5 µM. Traces shown are background corrected and indicate 0.25 µM and 2.5 µM of each tested sample. Starting time of analyte injection is given (0 seconds), contact time was limited to 120 seconds, while dissociation was monitored over 20 minutes. (D) A Biacore SA sensor chip was used to immobilize 5'-biotinylated DNA fragments comprising the identified binding sites of YehT and YpdB respectively. In a second step purified 6His-tagged RRs were injected forming a transient DNA-protein complex, before 6His-YehS was probed as analyte (0.01 µM to 5 µM). Traces shown are background corrected and indicate addition of 2.5 µM YehS. Starting time of analyte injection is given (0 seconds), contact time was limited to 120 seconds, while dissociation was monitored over 10 minutes. To probe phosphorylated RR derivatives, protein was incubated for 60 minutes with 50 mM Na^+/Li^+-acetylphosphate.

concentrations ≤ 1µM. No significant differences were found for the affinities of YehT-D54E or YpdB-D53E, respectively (data not shown).

To avoid hitherto observed effects we established our second approach the other way round by changing analyte and ligand, as we were unable to detect any interaction for YehS dimerization. For this experiment double-stranded 5'-biotinlyated DNA fragments comprising

the YehT- or YpdB-binding site were coupled to a Biacore SA sensor chip. In subsequent steps we captured the corresponding RRs or variants via specific protein-DNA interaction to the chip surface, before 6His-YehS was probed as analyte. This setup has successfully been used several times, e.g. for the quantification the KdpD/KpdE/UspC interaction in *E. coli* (Heermann *et al.*, 2009) or for the DNA-binding kinetics of two LytTR RRs from *Lactobacillus plantarum* C11 (Straume *et al.*, 2009). Binding of the RR to its cognate DNA fragment was carried out for 60 seconds. To reduce the rate of multimerized RR proteins, the immobilized protein-DNA complex was washed for 60 seconds. The ligand, ranging from 0.01 to 5 µM, was injected for 120 seconds, followed by 10 minutes of dissociation. All experiments were performed after complete regeneration of the SA chip surface to ensure comparable SPR measurements. Using this approach we initially measured the affinities of the protein-DNA interactions for the RRs to their target promoters. K_D values were determined between 110 and 250 nM and fit therefore very well to the K_D values described earlier (Kraxenberger *et al.*, 2012). But, as a result of rapid association and dissociation events, the observed protein-DNA complex demonstrated very transient binding properties. With the addition of 6His-YehS we clearly identified the prior observed interaction between the ligand and the protein-DNA complex, but we were not able to define K_D values precisely. However, a closer look to the SPR traces indicated an alteration within the binding properties of YehS upon RR phosphorylation (Fig. 5.3D1/D2). This effect results in a more gentle dissociation and therefore a higher affinity between YehS and the phosphorylated RR derivatives, which was not detected for the wild-type RRs or the phosphorylation independent derivatives. Phosphorylation-dependent protein-protein interactions provide the foundation for a multitude of intracellular signal transduction pathways (Shaywitz *et al.*, 2002). Given that fact and in accordance with the results from the BACTH screen, it is fair to assume, that the phosphorylation of the RRs might influence the YehS-mediated dynamics of protein-protein interactions in vivo.

Stimulus-response analysis of the YehU/YehT and YpdA/YpdB system. To gain insight into the expression pattern of *yjiY* and *yhjX* and the role of the network in vivo, transcriptional fusions of both defined promoters ($P_{yjiY\ -212/+88,}$ $P_{yhjX\ -264/+36}$) and the luciferase *luxCDABE* operon were constructed (plasmids pBBR *yjiY-lux,* pBBR *yhjX-lux*) (Kraxenberger *et al*., 2012, Fried *et al*., 2012). *E. coli* MG1655, E. coli MG 2 (Δ*yehUT*), *E. coli* MG20 (Δ*ypdABC*) and *E. coli* MG 30 (Δ*ypdABC* Δ*yehUT*) were transformed with this plasmids. Growth and luminescence (as a measure of *yjiY* or *yhjX* expression) under aerobic conditions were monitored in different inducing/repressing media over time. All tested strains showed comparable growth patterns with respect to different carbon sources.

Media containing peptides (such as e.g. Cas amino acids, tryptone, NZ-aa protein hydrolysate) as carbon sources are efficient inducers for *yjiY* (Kraxenberger *et al*., 2012) but

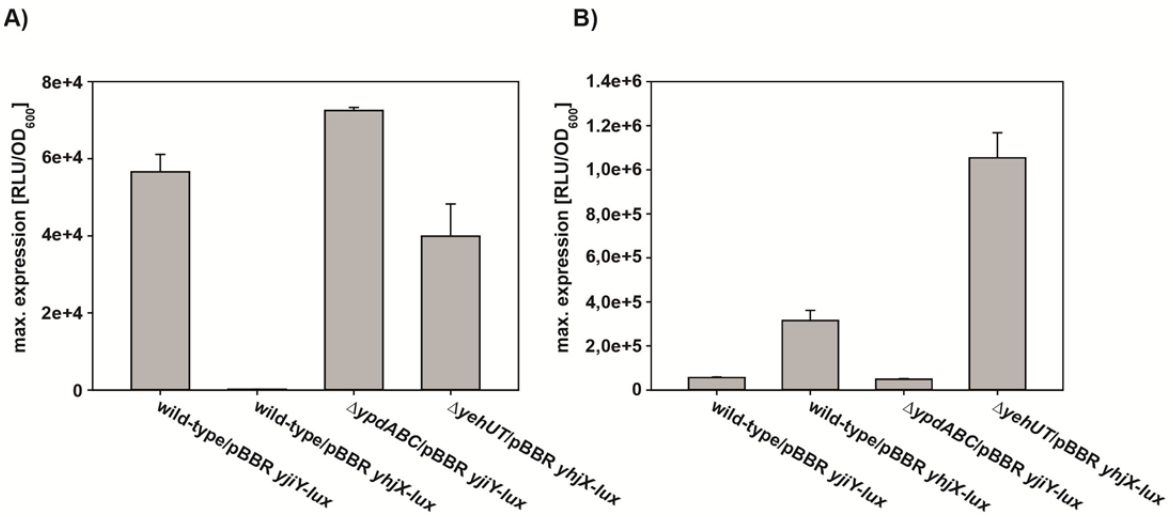

Fig. 5.4 Robustness to changing environmental conditions by TCS network integrity. A luciferase-based reporter assay was used to determine the pattern of *yjiY* and *yhjX* expression. Bacteria were cultivated under aerobic conditions, and growth and activity of the reporter enzyme luciferase were determined continuously. *E. coli* MG1655, MG20 (Δ*ypdABC*) and MG2 (Δ*yehUT*) were transformed with either pBBR *yjiY-lux* or pBBR *yhjX-lux* and grown in M9 minimal medium supplemented with Cas amino acids [0.4%] (A) or pyruvate [20 mM] (B), respectively. The maximal luciferase activity normalized to an optical density of 1 (RLU/OD_{600}) was used as a measure of the degree of induction of *yjiY* or *yhjX,* respectively. All experiments were performed at least three times, and the error bars indicate the standard deviation of the mean.

repress *yhjX* (Fried *et al*., 2012). So we got interested, if deletion of *yehUT,* resulting in constitutive repression of *yjiY,* is also affecting *yhjX* induction. In strain MG2 (Δ*yehUT*)/pBBR *yhjX-lux* the maximal luciferase activity was determined and used as an indicator for the degree of induction of *yhjX* (Fig. 5.4A). Interestingly, *yhjX* was expressed in M9 media with peptides as sole carbon source (Fig. 5.4A). Here, derepression started when the population was in mid-exponential growth phase. No induction of *yhjX* was observed in the *yehUT ypdABC* double deletion mutant MG30 (data not shown). In comparison to wild-type *E. coli* deletion of *ypdABC* had no effect on *yjiY* expression, indicating a subordinated role of this system.

Pyruvate is an efficient inducer for *yhjX* and induces *yjiY* 10 fold weaker. So, we tested if deletion of *yehUT* is affecting *yhjX* expression in vivo. In strain MG2 (Δ*yehUT*)/pBBR *yhjX-lux* the maximal luciferase activity was determined and used as an indicator for the degree of induction of *yhjX.* Deletion of *yehUT* resulted in a 3.3 fold increase of *yhjX* induction (Fig. 5.4B) compared to wild-type *E. coli*. Again, no induction of *yhjX* was observed in the *yehUT ypdABC* double deletion mutant MG30 (data not shown). In addition, deletion of *ypdABC* compared to wild-type *E. coli* had no effect on *yjiY* expression. Thus, it can be speculated that the YehU/YehT systems is superior to the YpdA/YpdB system by the coordination of a response. Such predominance of a HK/RR system has been already described in *P. aeruginosa*. Here, the HK GacS is superior to the HKs LadS and RetS (Gooderham &

Hancock, 2009). In addition, YehS, an accessory protein of the *yehUT* genomic neighborhood, interacts via direct protein-protein contacts with YehU, YehT, YpdA and YpdB. One might speculate that YehS mediates network integrity. However the exact, physiological role of YehS is up to now unknown. Neither deletion of *yehS,* nor overproduction of the corresponding gene product affected *yjiY* or *yhjX* expression significantly. Nevertheless, in most cases when both LytS/LytTR-like TCSs are present, YehS co-occurs indicating physiological advantage of YehS interactions.

Taken together, these results indicate that the network formation allows a physiological coordination of both systems to the sensed substrate and gives the systems a biological robustness.

Influence of carbon storage regulator A on *yjiY* and *yhjX*. Carbon storage regulator (CsrA) is an RNA binding protein that regulates gene expression post-transcriptionally by affecting ribosome binding and/or mRNA stability (Babitzke & Romeo, 2007). Members of the CsrB family of noncoding regulatory RNA molecules (*E. coli*: CsrB, CsrC) contain multiple CsrA binding sites and function as CsrA antagonists by sequestering this protein (Babitzke & Romeo, 2007). Depending on the particular organism, the Csr system participates in global regulatory circuits that control central carbon flux, the production of extracellular products, cell motility, biofilm formation, quorum sensing and/or pathogenesis (Romeo *et al.*, 2012). Previous studies demonstrated that *yehS* is regulated by CsrA (Edwards *et al.*, 2011). In addition synthesis of CstA, a putative peptide transporter (Schultz & Matin, 1991) which demonstrates a high sequence identity (62%) and similarity (76%) to YjiY, is subject to CsrA regulation (Dubey *et al.*, 2003) by blocking the ribosome access to the *cstA* transcript.

The 5´untranslated mRNA leader structures of *yjiY* and *yhjX* contain in comparison to the well described CsrA binding site (Edwards *et al*., 2011), two or one, respectively, imperfect CsrA binding sites. To gain insights into the role of the Csr system on *yhjX* and *yjiY* translational luciferase based fusions (plasmids pBBR *yjiY'-'lux*, pBBR *yhjX'-'lux*) were constructed and expression analysis upon overproduction of CsrA, CsrB, CsrC and the empty vector (negative control) was performed. *E. coli* LMG194 (Guzman *et al.*, 1995) was chosen as host strain because it can uptake but not metabolize L-arabinose. Overproduction of CsrB (19.2 fold) and CsrC (13.9 fold) resulted in an increased *yjiY* expression, whereas overproduction of CsrA 2.1 fold repressed (Fig. 5.5A, upper panel). In parallel, deletion of *csrA* resulted in constitutive induction of *yjiY* (Fig. 5.5A, lower panel). In contrast, induction of *yhjX* was 11.3 fold increased when CsrA was overproduced (Fig. 5.5B, upper panel). Overproduction of CsrB (9.2 fold) and CsrC (1.5 fold) resulted in a reduced *yhjX* repression. The deletion of *csrA* totally abolished *yhjX* expression (Fig. 5.5B, lower panel). These data

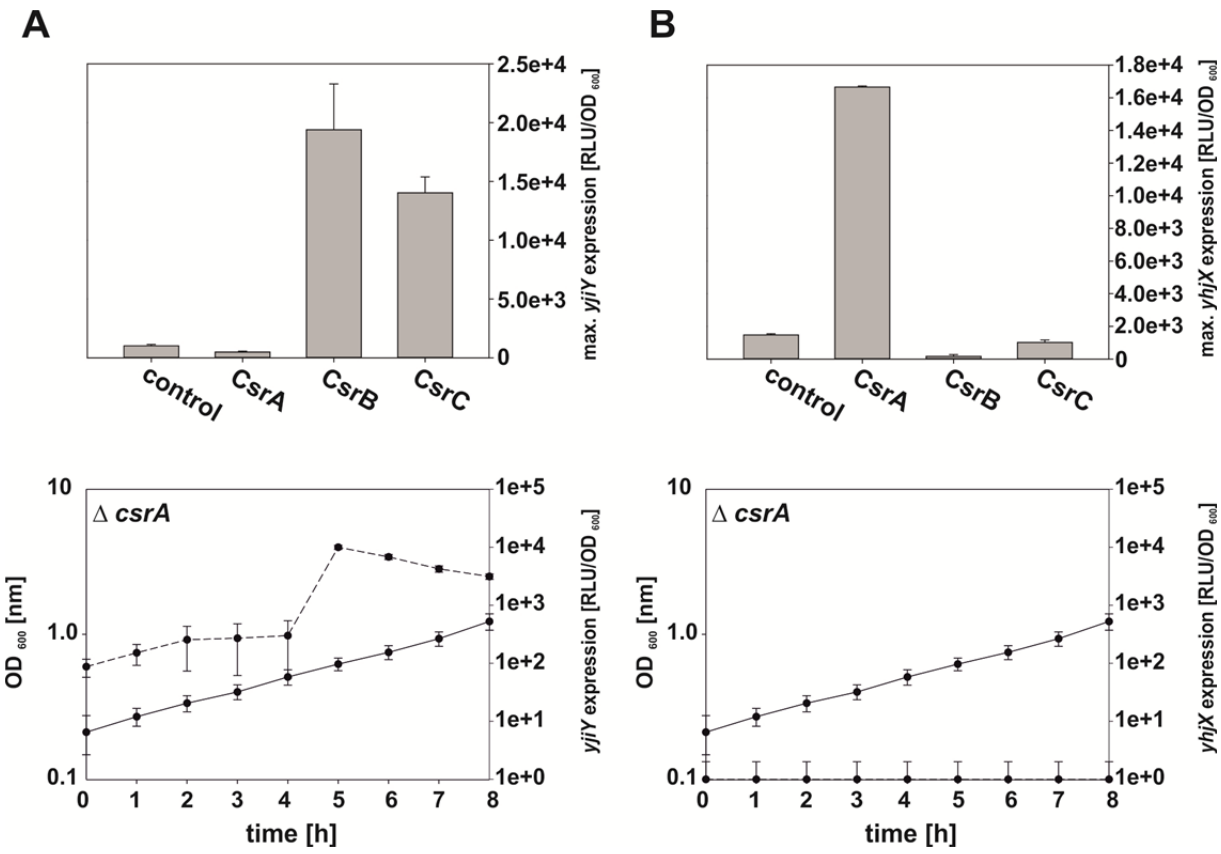

Fig. 5.5 Carbon storage regulator A is influencing *yjiY* and *yhjX* post-transcriptionally. A luciferase-based reporter assay was used to determine the pattern of *yjiY* (A) and *yhjX* (B) expression. Bacteria were cultivated under aerobic conditions, and growth and activity of the reporter enzyme luciferase were determined continuously. Upper panels) *E. coli* LMG194 was transformed with pBBR *yjiY´-´lux* (A) or pBBR *yhjX´-´lux* (B), respectively and grown in LB medium supplemented with L-arabinose [0.2%] (upper panel). In addition, these reporter strains contained plasmid pBAD24 (control), pBAD24-*csrA*, pBAD24-CsrB or pBAD24-CsrC. Lower panels) *E. coli* MG31(Δ*csrA*) was transformed with pBBR *yjiY'-'lux* (A) or pBBR *yhjX'-'lux* (B), respectively and grown in LB medium. The maximal luciferase activity normalized to an optical density of 1 (RLU/OD_{600}) was used as a measure of the degree of induction of *yjiY* or *yhjX,* respectively. All experiments were performed at least three times, and the error bars indicate the standard deviation of the mean.

indicate that CsrA blocks the 5´untranslated mRNA of *yjiY* and promotes stability of *yhjX* 5´untranslated mRNA. Such a bias-regulation by CsrA is already known, as it positively post transcriptionally regulates the flagellar master regulator genes *flhDC* (Wei *et al.*, 2001), but represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesion in *E. coli* (Wang *et al.*, 2005). Further RNA binding studies have to verify a direct binding of CsrA to *yjiY* and *yhjX* mRNAs.

Taken together, our data indicate the participation of the Csr system as additional checkpoint in the LytS/LytTR signaling network of *E. coli*.

The LytS/LytTR-type HK/RR signaling network in *E. coli*. In this study we identified the YehU/YehT YpdA/YpdB TCS signaling network of *E. coli* (summarized in Fig. 5.6): We could demonstrate a coordinated expression of the genes *yjiY, yhjX* and *yehS*. Furthermore, we showed direct protein-protein interactions between the TCS components and an accessory protein YehS. A physiological connection between both systems was demonstrated, allowing a robust cellular response to different environmental conditions.

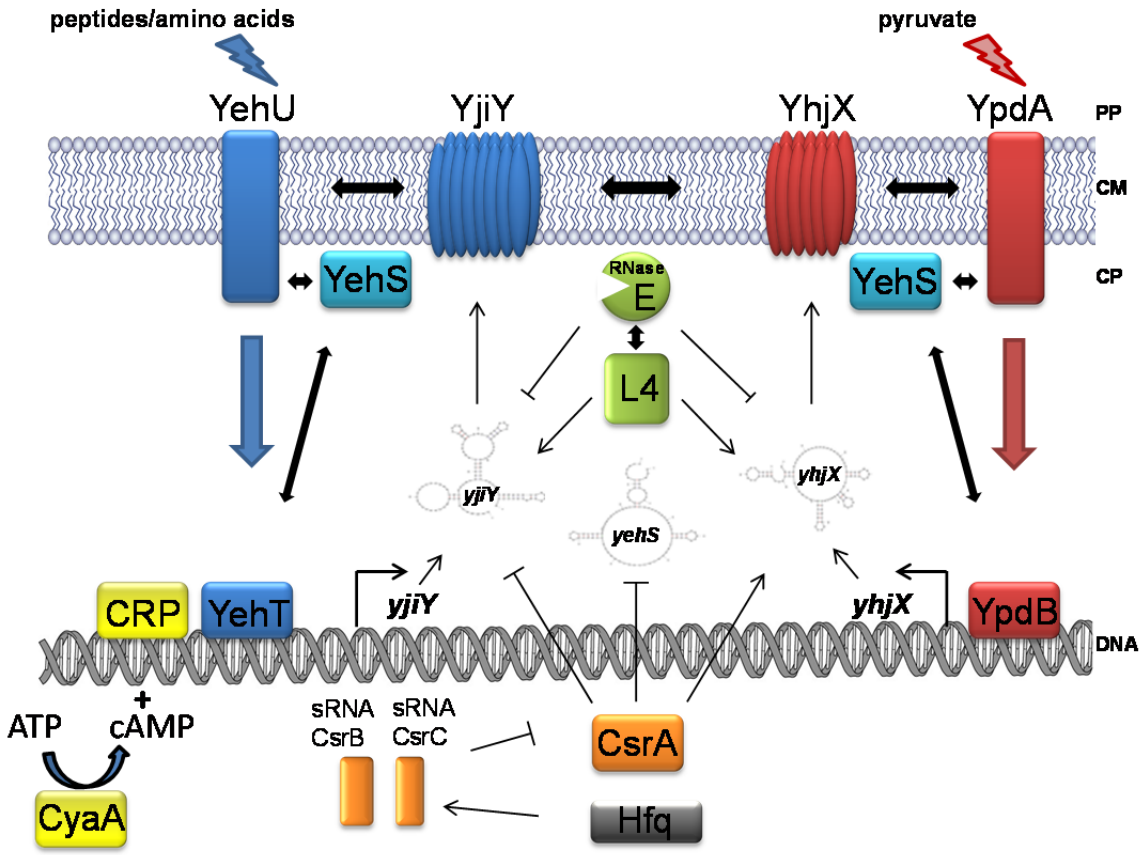

Fig. 5.6 The YehU/YehT and YpdA/YpdB signaling network in *Escherichia coli*. Activating (↑) or inhibitory (⊥) effects based on (Edwards et al., 2011, Kraxenberger et al., 2012, Singh et al., 2009, Fried et al., 2012) and this manuscript are marked. Protein-protein interactions are marked by double arrows. Membrane proteins are integrated in the cytoplasmic membrane and DNA-binding proteins are bound to the DNA. Arrows (↱) mark the transcription start sites. See text for details. PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm.

Interconnectivity between two or more TCSs has been described in different forms recently (Jung *et al.*, 2012). In *Salmonella enterica* e.g. the TCSs PhoP/PhoQ and PmrA/PmrB are coordinated via a connector protein PmrD to mediate polymyxin B resistence (Kox *et al.*, 2000), whereas in *E. coli* the TCS-mediated regulation to acid resistance connects two single signal transduction pathways (Eguchi *et al.*, 2011).

Under inducing conditions the two HKs YehU (peptides/amino acids) and YpdA (pyruvate ≥250 µM) sense the stimulus/stimuli. Whether the flux of information is achieved by phosphorylation or mediated by protein-protein interactions is under current investigation. Nevertheless, activated RR homodimerizes and binds to the corresponding target gene promoter regions (YehT→ P_{yjiY}; YpdB→ P_{yhjX}) resulting in *yjiY* and *yhjX*, respectively, expression. In addition, *yjiY* induction underlies cAMP/CRP regulation (Kraxenberger *et al.*, 2012). Before YjiY, a putative peptide transporter, or YhjX a putative MFS transporter are produced several regulatory mechanisms on mRNA level occur. CsrA, carbon storage regulator A, can probably bind the 5´untranslated leader mRNAs of *yjiY, yhjX* (see chapter above) and *yehS* (Edwards *et al.*, 2011). CsrA blocks in the case of *yjiY* or promotes in the

case of *yhjX* the ribosome access and thereby modifies translation initiation. The small RNAs CsrB and CsrC, which are activated by the BarA/UvrY TCS and require the molecular chaperone Hfq, antagonize CsrA by sequestration (Romeo *et al*., 2012). Thus, CsrB and CsrC positively post-transcriptionally regulate *yjiY* or repress *yhjX*. Furthermore, *yjiY* and *yhjX* transcript degradation underlies the interplay between the ribosomal protein L4 and RNase E (Singh *et al*., 2009). L4 protein binds the catalytic domain of RNase E and inhibits target specific cleavage resulting in an increase of *yjiY* and *yhjX* mRNA (Singh *et al*., 2009). Protruding the RNA regulatory checkpoints, YjiY and YhjX are produced and membrane integrated. Here, interactions between both transporters and both HKs can take place.

In conclusion, the LytS/TR like regulatory network of *E. coli* was identified as another player in the complex carbon control system. Further experiments will concentrate on the linkage between these systems to obtain better insights into its internal regulation, the physiological function and the characterization of the transport/regulatory proteins.

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6 Histidine kinases and response regulators in networks

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Abstract

Two-component systems, composed of a histidine kinase (HK) and a response regulator (RR), are the major signal transduction devices in bacteria. Originally it was thought that these two components function as linear, phosphorylation-driven stimulus-response system. Here, we will review how accessory proteins are employed by HKs and RRs to mediate signal integration, scaffolding, interconnection and allosteric regulation, and how these two components are embedded in regulatory networks.

Full-text article:

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7 Concluding Discussion

Among two-component signal transduction systems, the LytS/LytTR HK/RR family regulates essential cellular functions in Gram-positive pathogenic bacteria (Galperin, 2008). By contrast, in Gram-negative bacteria like *Escherichia coli*, little was known about the signaling mechanisms of the corresponding YehU/YehT and YpdA/YpdB LytS/LytTR-like systems.
Employing comprehensive reporter tools (Chapter 2), elaborate studies on the YehU/YehT TCS were performed focusing on YehT-dependent gene regulation (Chapter 3). Based on these findings, the mode of signal perception, signal integration, and alteration of gene expression by the YpdA/YpdB TCS was characterized (Chapter 4). Further, a state-of-the-art picture of network formation in TCSs was presented (Chapter 6). Concluded from these data, analysis of protein-protein interactions and transcriptional regulation demonstrated a physiologic interplay between both of the LytS/LytTR-like systems in *E. coli* (Chapter 5). Altogether, the results of this thesis permit the establishment of a comprehensive model of LytS/LytTR-like regulation in *Escherichia coli*.

The molecular hub of LytS/LytTR-like two-component systems in Escherichia coli

In the course of this thesis, the target genes, *yjiY* of the YehU/YehT system, and *yhjX* of the YpdA/YpdB system, were identified (Chapters 3 and 4). A transient expression of both genes was determined in the mid-exponential growth phase (Chapter 5). Moreover, comprehensive carbon source evaluation studies revealed the inducing stimuli for both systems. Peptides and amino acids are preferentially sensed by the YehU/YehT system, whereas pyruvate is sensed by the YpdA/YpdB system. The similar induction profiles of both systems and the identification of pyruvate as stimulus for the YpdA/YpdB system suggested that both systems regulate in the scavenging phase of *E. coli* (Chapter 4). Moreover, protein-protein interaction studies demonstrated that both systems, their corresponding target gene products, YjiY, a putative peptide transporter, and YhjX, an uncharacterized member of the Major Facilitator Superfamily, and an accessory protein, YehS, are embedded in a complex signaling network (Chapter 5).
In the following sections, a comprehensive model of YehU/YehT and YpdA/YpdB regulation is presented (Fig. 7.1), and the molecular details of the network components are conclusively discussed.

7.1 A comprehensive model for YehU/YehT and YpdA/YpdB regulation in *Escherichia coli*

Under scavenging conditions, the two membrane-integrated HKs, YehU and YpdA, sense the stimulus/stimuli. YehU putatively perceives peptides/amino acids (Chapter 3), while YpdA senses pyruvate in concentrations higher than 250 µM (Chapter 4). Upon stimulus perception, YehU and YpdA transduce the signal to their cognate RRs, YehT or YpdB, respectively (Fig. 7.1). Whether the flux of information is achieved by phosphorylation or mediated by protein-protein interactions is under current investigation (see Chapter 7.6). Nevertheless, the activated RR binds to the corresponding target gene promoter regions (YehT→ P_{yjiY}; YpdB→ P_{yhjX}), resulting in the expression of *yjiY* and *yhjX*, respectively. In addition, *yjiY* induction is dependent on cAMP/CRP regulation (Chapter 3), an attribute of the scavenging phase (Peterson *et al.*, 2005). *yjiY* expression is significantly reduced or abolished in a *cyaA* or a *crp* deletion mutant.

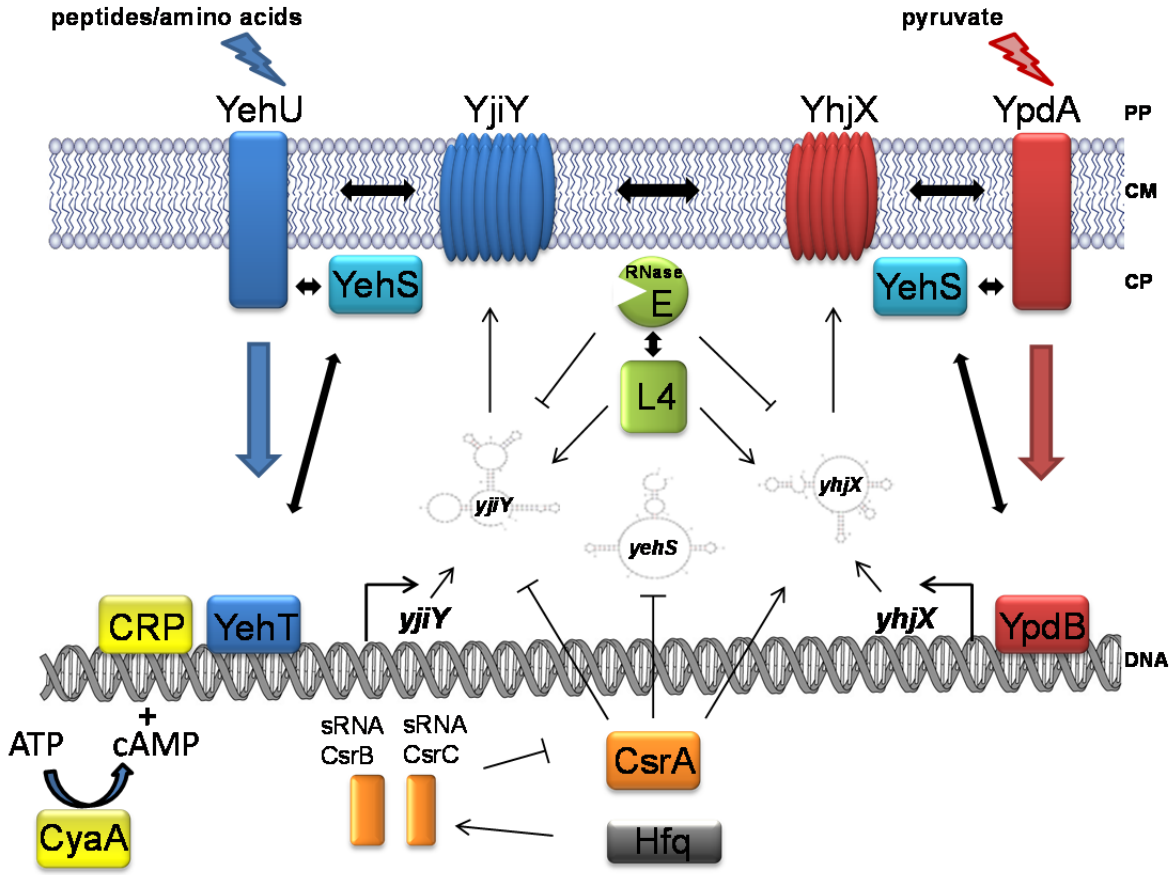

Fig. 7.1 The YehU/YehT and YpdA/YpdB signaling network in *Escherichia coli*. Activating (↑) or inhibitory (⊥) effects based on (Chapter 3, 4, and 5, Edwards *et al.*, 2011, Singh *et al.*, 2009) are marked. Protein-protein interactions are labeled by double arrows. Membrane proteins are integrated in the cytoplasmic membrane and DNA-binding proteins are bound to the DNA. Arrows (↱) mark the transcription start sites. See text for details. PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm.

Moreover, by mutation of the CRP consensus sequence in the *yjiY* promoter, the induction of *yjiY* occurs to the same degree as in the *cyaA* mutant (Chapter 3). Before YjiY, a putative peptide transporter, or YhjX, a putative MFS transporter, is produced, several regulatory mechanisms occur on the mRNA level:

Carbon storage regulator (CsrA) is an RNA binding protein that regulates gene expression post-transcriptionally by affecting ribosome binding and/or mRNA stability (Babitzke & Romeo, 2007). Members of the CsrB family of noncoding regulatory RNA molecules (*E. coli*: CsrB, CsrC) contain multiple CsrA binding sites and function as CsrA antagonists by sequestering this protein (Fig. 7.1) (Babitzke & Romeo, 2007). Depending on the particular organism, the Csr system participates in global regulatory circuits that control central carbon flux, the production of extracellular products, cell motility, biofilm formation, quorum sensing and/or pathogenesis (Romeo *et al.*, 2012). Previous studies demonstrated that *yehS* mRNA of *E. coli* is regulated by CsrA (Edwards *et al*., 2011). In addition, synthesis of CstA, a putative peptide transporter (Schultz & Matin, 1991) which possesses a high sequence identity (62%) and similarity (76%) to YjiY, is subject to CsrA regulation (Dubey *et al.*, 2003). CsrA blocks the ribosome's access to the *cstA* transcript (Dubey *et al*., 2003). Therefore, several translational expression studies upon overproduction of the components of the Csr system were performed (Chapter 5). They revealed that CsrA can probably bind to the 5´ untranslated leader mRNAs of *yjiY* and *yhjX*. CsrA blocks the ribosome's access to *yjiY* mRNA, and thus modifies translation initiation (Fig. 7.1). In contrast, CsrA promotes ribosome's access to *yhjX* mRNA (Fig. 7.1). Overproduction of CsrA resulted in elevated expression. In addition, *yjiY* and *yhjX* expression was analyzed upon deletion of *csrA*. Concordantly, deletion resulted in a constitutive *yjiY* expression, whereas *yhjX* was not expressed (Chapter 5).

The small RNAs CsrB and CsrC, which are activated by the BarA/UvrY TCS and require the molecular chaperone Hfq for correct folding, antagonize CsrA by sequestration (Fig. 7.1) (Romeo *et al*., 2012). Thereby, CsrB and CsrC promote *yjiY* and inhibt *yhjX* expression (Fig. 7.1, Chapter 5). However, *yjiY* or *yhjX* expression is reduced or prevented in an *hfq* deletion mutant (Behr, 2012; unpublished data). This indicates that either Hfq is important for folding of *yjiY* and *yhjX* mRNAs (in addition to the folding of CsrA and CsrB), or that a so far unknown regulatory RNA is involved in signaling. Interestingly, the physiological stimulus of the BarA/UvrY TCS is supposed to be acetate (Chavez *et al.*, 2010). In overflow metabolism, acetate also accumulates and a metabolic intermediate is always pyruvate. Moreover, *E. coli* grows faster on pyruvate compared to acetate (Holms, 1996), indicating that pyruvate is the more favorable C-source. With increasing extracellular acetate concentrations, the BarA/UvrY TCS is activated and the small RNAs CsrB and CsrC are produced, thereby antagonizing CsrA. Thus, levels of *yjiY* are increased, whereas levels of *yhjX* mRNA are

decreased. Concordantly, acetate as sole C-source in culture media was an efficient inducer for the YehU/YehT system, whereas in the case of YpdA/YpdB, *yhjX* was not expressed (Chapters 3 and 4).

Furthermore, *yjiY* and *yhjX* transcript degradation is dependent upon the interplay between the ribosomal protein L4 and RNase E (Fig. 7.1) (Singh *et al*., 2009). Here, it was shown that RNase E binds and degrades *yjiY* and *yhjX* transcripts (Chapter 5). However, when the L4 protein binds the catalytic domain of RNase E and inhibits target-specific cleavage, there is a resultant increase in *yjiY* and *yhjX* mRNA (Singh *et al*., 2009). After passing through the RNA regulatory checkpoints, YjiY and YhjX are produced and integrated into the membrane. Here, interactions between both transporters and both HKs can occur, which are probably involved in signal perception and output responses. At the moment, the modes of transport and energization, as well as the specific substrate, can only be speculated upon. However, it would be feasible that molecules that are highly abundant in the scavenging phase could be responsible.

7.2 Regulation in the molecular switch between overflow metabolism and carbon starvation

In a term called overflow metabolism exponentially growing bacteria secrete by-products (e.g. pyruvate) into the medium to avoide metabolic bottlenecks (Paczia *et al*., 2012). However, when the remaining C-source is consumed, excreted by-products (e.g. pyruvate) are taken up rapidly to continue growth in a mechanism called scavenging. Therefore, bacteria have to adapt accordingly. Scavenging is a well-known characteristic of mid-exponential growth, where cells try to exhaust remaining nutrients in their environment to prevent stationary growth. The finding that the expression of *yjiY* and *yhjX* is coordinated in mid-exponential growth phase (Chapter 3, 4 and 5) provides the first evidence to support that the YehU/YehT and the YpdA/YpdB systems are involved in *E. coli* scavenging.

A switch to carbon starvation has been suggested to involve a two-stage starvation protocol (Peterson *et al*., 2005): The first response is scavenging, a process in which the production of proteins that forage for the limiting nutrient is increased (Peterson *et al*., 2005). Global scavenging regulons, including cAMP/CRP, which allow the use of alternative carbon sources, or the TCSs, NtrB/NtrC and PhoR/PhoB, which control scavenging for nitrogen and phosphorus, respectively, are operating during the first part of this response (Wanner, 1996). When scavenging fails, cells starve and switch to the “dormant mode”, better known as the stationary phase (Peterson *et al*., 2005). Subsequently, a global reprogramming of the cellular gene expression profile mediated by the stationary phase sigma factor, RpoS, occurs (Hengge-Aronis, 2002). The master regulator RpoS controls, among other essential functions, the levels of bis-(3’-5’)-cyclic dimeric guanosine monophosphate (c-di-GMP),

which is crucial to the motile-to-sedentary "lifestyle" switch (Hengge, 2009). Such radical changes have to be tightly controlled. Indeed, the maximal pyruvate concentration during growth correlates with mid-log growth phase and rapidly decreases during the scavenging phase (Chapter 4). Moreover, expression analysis and the presence of a putative quorum sensing-like molecule suggest that at this point of growth, YjiY is operating. This in turn could mean that the YjiY and YhjX and the corresponding YehU/YehT and YpdA/YpdB systems operate at the interface to stationary growth phase to prevent the "dormant mode" and/or generates an appropriate response to changing environmental conditions.

7.3 The importance of YjiY and YhjX

Since a correlation between inducing stimuli, expression profiles and participation in scavenging response between the YehU/YehT and YpdA/YpdB systems was observed, we became interested in their physiologic relevance. In the following chapter, a combination of functional, biochemical and regulatory aspects of the putative peptide transport protein, YjiY, and the major facilitator superfamily transporter, YhjX, are provided.

The putative peptide transporter YjiY

Enteric bacteria such as *E. coli*, *Shigella flexneri*, and *Salmonella typhimurium,* prefer the intestine of warm-blooded animals as their natural habitat (Bearson *et al.*, 1997). Notably, the gastric juice contains high levels of amino acids, such as alanine, proline, and serine, as well as pyruvate (Nagata *et al.*, 2003, Nagata *et al.*, 2007). Under these conditions, maximal *yjiY* expression was identified (Chapter 3). By combining these facts, one might speculate that the presence of YjiY may allow for the propagation and colonization of these bacteria under such ideal conditions. In addition, cells that already harbor YjiY in their membrane could have a growth advantage over other bacteria during colonization of such organs as the intestine. Likewise, *yjiY* expression was induced in vivo in avian pathogenic *E. coli* (APEC) during the infection process in chicken liver and spleen (Tuntufye *et al.*, 2012). Both of these organs are known to contain high levels of amino acids (Brosnan, 2000, Mebius & Kraal, 2005).

Another second attractive hypothesis is that YjiY and the YehU/YehT system might play a role in quorum sensing of *E. coli*. Expression profiles of *yjiY* demonstrated that this system always regulates at a certain cell-density (OD_{600} ~ 0.6 - 1.0; Chapters 3 and 5). Moreover, the addition of supernatants containing the putative quorum sensing molecule to cells induced *yjiY* expression in reporter assays. The presence of quorum sensing in *E. coli* was demonstrated, but the small molecule secreted and its corresponding system remained elusive (Surette & Bassler, 1998). However, the maximal secretion of the secreted molecule occurred in the mid-exponential growth phase and was abolished in the stationary growth phase (Surette & Bassler, 1998). Taking these data into account it can be suggested that

YjiY and the YehU/YehT system are probably involved in the quorum sensing process of *E. coli*. It is known that, in Gram-positive bacteria, peptides are the predominant cell-to-cell communication molecules. In Staphylococci genes encoding for the autoinducing peptide (AIP), the transporter AgrB and the LytS/LytTR-like AgrC/AgrA TCS are organized in the *agrBDCA* operon, and expressed constitutively at a low level (Geisinger *et al.*, 2008). AIP is the inducing ligand for the HK AgrC, which activates the RR AgrA, upon a certain AIP-threshold concentration. Activated AgrA binds its own promoter and the RNAIII promoter, resulting in up-regulation of genes involved in virulence (Novick & Geisinger, 2008). In addition, inhibitory non-cognate/heterologous AIP from other species compete with the cognate AIP for receptor binding and modulate its activation. Recently, it became evident that small peptides are also excreted by Gram-negative bacteria (Fozo *et al.*, 2008). Consequently, it may be possible that the YehU/YehT TCS and the transport protein YjiY participate in a similar process. Further studies are required to identify the function of YjiY, its mode of transport (importer or exporter), its specific substrate, and the mode of energization. The participation of YjiY in peptide/amino acid uptake or the export of some still unknown quorum-sensing molecule can at the moment only be speculated upon.

YhjX – a transporter of the major facilitator superfamily

Expression of *yhjX* is induced by the YpdA/YpdB-system in response to pyruvate and under the metabolic conditions of the Entner-Doudoroff pathway (gluconate, glucuronate). Pyruvate (pK_a ~ 2.50), as a acid, cannot diffuse through biological membranes (Halestrap, 1975). Likewise, active transport of pyruvate was demonstrated but the transport proteins remained elusive (Lang *et al.*, 1987). The pyruvate-coordinated *yhjX* expression profile suggested a physiological relevance of increased extracellular pyruvate levels in combination with YhjX. Hence, one might speculate that the YhjX transport protein and the YpdA/YpdB TCS are involved in pyruvate utilization. Pyruvate is a central intermediate in carbon metabolism and is involved in processes such as glycolysis, gluconeogenesis, fatty acid synthesis, amino acid synthesis and fermentation (Wolfe, 2005). However, in a process called overflow metabolism, pyruvate gets secreted to avoid metabolic bottlenecks (Holms, 1996). Here, pyruvate, besides acetate, is the major component of the exometabolome in a broad range of organisms (Paczia *et al.*, 2012). As pyruvate is the key metabolite between respiration/fermentation and a precursor to several macromolecules, its cellular concentration has to be tightly controlled (Vemuri *et al.*, 2006). Under non-favorable growth conditions, when the remaining C-source is consumed, excreted by-products (e.g. pyruvate) are taken up rapidly to continue growth (Paczia *et al.*, 2012). Therefore, the external and internal pyruvate levels have to be sensed precisely to generate an adequate response in order to adapt perfectly. Thus, it could be suggested that YhjX may be involved in pyruvate utilization. Relevance of pyruvate utilization meditated by LytS/LytTR-like TCS in *S.*

epidermidis was recently identified (Zhu *et al.*, 2010). Deletion of *lytSR* resulted in the already known defects in murein hydrolase activity and bacterial cell death regulation. Moreover, bacterial growth was defective when pyruvate was used as the sole C-source.

However, the function of YhjX is unknown thus far. Based on sequence similarities to the oxalate:formate antiporter, OxlT, in *Oxalobacter formigenes,* a function of YhjX as a carboxylate exchange system had been postulated but was never proven (Pao *et al.*, 1998, Keseler *et al.*, 2009). Participation of YhjX in carboxylate uptake or in the export of some thus-far-unknown molecule can only be speculated on. Further studies are required to identify the function of YhjX, its mode of transport (importer or exporter), its specific substrate and the mode of energization. Interestingly, preliminary transport experiments demonstrate a proton-dependent pyruvate accumulation in cells harboring YhjX (Raba, 2012; unpublished data), providing first evidence for active transport of pyruvate by YhjX.

Another attractive hypothesis is that pyruvate sensing by the YpdA/YpdB TCS and subsequent uptake of pyruvate by YhjX could provide benefits in adaptation to the enteric environment. This is further supported by the fact that pyruvate is one of the major components of gastric juice and is present in the intestine (Nagata *et al*., 2003, Hooper *et al.*, 2002). Therefore, improved sensing of pyruvate in the gut could lead to adaptative advantages in intestinal colonization, as pyruvate is a major component also found in the intestine (Hooper *et al*., 2002). Concordantly, it became evident that eukaryotic colon cells excrete pyruvate to evade cell death (Thangaraju *et al*., 2009). Thus, high extracellular levels of pyruvate could probably be a stimulus for bacterial cells to adapt to the intestinal environment.

7.4 YehS – technical support of YehU/YehT and YpdA/YpdB signaling

YehS, an accessory protein of the *yehUT* genomic neighborhood, interacts via direct protein-protein contacts with YehU, YehT, YpdA and YpdB (chapter 5). However the exact physiological role of YehS is unknown. Neither deletion of *yehS,* nor overproduction of the corresponding gene product, affected *yjiY* or *yhjX* expression significantly (Chapter 5). Nevertheless, in genome sequences which encode both LytS/LytTR-like TCSs, *yehS* is also found, suggesting that there is a physiological advantage for YehS interactions. Interconnectivity between two or more TCSs has been described in different forms (Chapter 6). In *Salmonella enterica* the TCSs, PhoP/PhoQ and PmrA/PmrB, are coordinated via a connector protein, PmrD, to mediate polymyxin B resistance (Kox *et al.*, 2000), whereas in *E. coli,* the TCS-mediated regulation to acid resistance connects two single signal transduction pathways via SafA (Eguchi *et al.*, 2011). Recently, an auxiliary protein complex, SaePQ, in *S. aureus,* was shown to activate the phosphatase activity of the corresponding HK SaeS

(Jeong *et al.*, 2012). Co-sensing, as described for the DcuS/DcuR system and the transport protein, DctA, (Witan *et al.*, 2012) could be also one mode of action, but seems unlikely as the deletion of *yehS* revealed no phenotype. For several systems, such as the GacS/GacA TCS in *P. aeruginosa,* network integrity and interaction with other HKs (LadS, RetS) is essential for virulence. Thus, it can be speculated that YehS increases network integrity.

7.5 Protein-protein interactions within the network

Protein-protein interaction studies demonstrated interactions between single network components. Using the bacterial two-hybrid system homo-oligomerization for the HK (YehU-YehU), the RRs (YehT-YehT, YpdB-YpdB) and the target gene products YjiY and YhjX (YjiY-YjiY, YhjX-YhjX) was determined (Chapter 5). In addition, homo-oligomerization of the RRs, YehT and YpdB, respectively, were validated via surface plasmon resonance measurements. In general, activated HKs and RRs operate as dimers (Capra & Laub, 2012). Similarly, our results suggest that YehT and YpdB bind as dimer to their cognate binding sites. Similar prototypical RR binding was demonstrated for several LytTR proteins like AgrA in *S. aureus* (Sidote *et al.*, 2008). Moreover, in vivo protein-protein interactions using the bacterial two-hybrid system demonstrated a homo-dimerization of YehT and YpdB, for several N- and C-terminal fusion proteins (Chapter 5). Taking these data into account, a head-to-head association of monomers can be speculated. For several RRs, this arrangement displays the predominant mode of DNA binding, as exemplified by the virulence gene regulator, BvgA, in *Bordetella pertussis* (Boucher *et al*., 2003).

By contrast, in bacterial two-hybrid protein-protein interaction studies also hetero-oligomerization between the HKs (YehU or YpdA) and both transport proteins (YjiY and YhjX) was determined (YehU-YjiY, YehU-YhjX, YpdA-YhjX, YpdA-YjiY) (Chapter 5). In addition, we identified protein-protein interactions between YjiY and YhjX (YjiY-YhjX), but not between the HKs YehU and YpdA. Hetero-oligomerization is observed in bacterial signaling but rather poorly understood (Gao *et al.*, 2008). In eukaryotes hetero-oligomerization of G-protein-coupled receptors plays a fundamental role in signaling (Overton *et al.*, 2005). Thus, it could be speculated that the hetero-oligomerization and complex formation of Lyt-like components is important for signaling and/or transport. Complex formation between several HKs and trigger transporters has been described by Tetsch & Jung (Tetsch & Jung, 2009). The interplay between sensor protein and (sensing-) transporter, is essential for regulation. However, deletion of *yjiY* and *yhjX* did not abolish sensing of the corresponding TCS (Chapter 3 and Chapter 4). This in turn could mean, that either the complex formation is important for correct positioning of the receptor and transporter, and/or that feedback regulation occurs on the substrate level. Interestingly, an interaction of a small membrane

protein, YohO, (Hemm *et al.*, 2008) with YehU, has already been demonstrated (Behr, 2011; unpublished data). Moreover, another small membrane protein, YpdK, is adjacently localized to the *ypdABC* operon (Chapter 4). One might speculate that these small membrane proteins interact with the systems components to stabilize the molecular hub of LytS/LytTR signaling systems.

7.6 Phosphorylation and alternative ways of signal transduction

The molecular details of the phosphorylation sites in the YehU/YehT and YpdA/YpdB systems were analyzed as part of this thesis. Although in vitro phosphorylation of both HKs (YpdA, YpdB) was very weak and phosphotransfer to the RRs (YehT, YpdB) was undetectable, our in vivo data indicate the functional importance of the phosphorylation sites. Substitution of the conserved phosphorylation sites (YehU-H382Q, YehT-D54N; YpdA-H371Q, YpdB-D53E) prevented phosphorylation and subsequent target gene expression in in vivo reporter assays. Consequently, gain of function substitution of the conserved aspartate with glutamate (YehT-D54E; YpdB-D53E) resulted in a phosphorylation-independent target induction. Originally, it was thought, that in signaling processes reversible phosphorylation of HKs and RRs are the major “signal-transmission” elements that control cellular responses. However, for some systems sequestration through protein-protein interactions rather than phosphorylation can also mediate the flux of information (O'Hara *et al.*, 1999). Such relevance of protein-protein interactions, even in the absence of a cognate RR, was demonstrated between two HKs and a developmental regulator, MrpC, in *M. xanthus* (Schramm *et al*., 2012). Interestingly, in the case of the YpdA/YpdB system, the presence of high concentrations of the stimulus activates the response regulator even when the crucial residue of the phosphorylation site of the HK (e.g. pyruvate and YpdA-H371Q) is substituted (Fried 2012; unpublished data). This could mean that protein-protein interactions in LytS/LytTR-like systems in addition to phosphorylation events are also of importance to signal transduction.

7.7 The diversity of LytTR regulators

Beyond the initial identification of *yjiY* and *yhjX* as target genes of the YehU/YehT and YpdA/YpdB systems, additional biochemical and structural approaches characterized the corresponding DNA binding motifs. The YehT-binding site, composed of two direct repeats of the motif, ACC(G/A)CT(C/T)A, separated by a 13-bp spacer, and the YpdB-binding composed of two direct repeats of the motif, GGCATTTCAT, separated by a 11-bp spacer, were identified and proven in the corresponding *yjiY* and *yhjX* promoter regions (Chapter 3,

Chapter 4 and Table 7.1). This is in contrast to Nikolskaya's and Galperin's postulation that the LytTR-like family ("litter") binding motif is an imperfect direct repeat of the consensus sequence, (T/A)(A/C)(C/A)GTTN(A/G)(T/G), separated by a 12-13 bp spacer (Nikolskaya & Galperin, 2002). The predicted motif was based on the upstream regulatory sequences of the *agr* locus and a regulatory RNA, RNAIII, (Koenig *et al.*, 2004) in *S. aureus* (Table 7.1). However, our findings suggest less motif conservation within the LytTR-like family. In line with our findings, the DNA-binding motifs of LytTR-like RRs dramatically differ among different species (Table 7.1) suggesting that there is a larger diversity among the different types of DNA-binding domains.

Since the corresponding binding sites were identified, we now focused on nucleotides bracketing the motifs. Strikingly, nucleotides adjacent to the binding sites were found to influence promoter activity. Thus, it is possible that the overall DNA-RR structure is important for regulation. Such a phenomenon is already described for the cAMP receptor protein CRP in *E .coli* (Hardwidge *et al*., 2002). Therefore, several nucleotides adjacent to the binding sites were substituted. Intriguingly, the substitution of central nucleotides within the spacer

Table 7.1 : DNA-binding motifs of LytTR-like regulators

protein	organism	binding sequence (5´-3´)	spacer length (bp)	regulated gene(s)	regulated process	reference
AgrA	*Staphylococcus aureus*	(T/A)(A/C)(C/A)GTTN(A/G)(T/G)	12-13	*agr* locus; RNAIII	virulence, peptide quorum sensing	(Koenig *et al*., 2004)
AlgR	*Pseudomonas aeruginosa*	CCGT(G/T)(C/G)(G/T)TC	-*	*fimU-pilVWXY1Y; hcnA; algD*	alginate production, type IV pilus function, and virulence	(Lizewski *et al*., 2004)
BlpR	*Streptococcus pneumoniae*	ATT(C/T)ANGANNT	10	*blp* operon	bacteriocin production, peptide quorum sensing	(de Saizieu *et al*., 2000)
BrsR	*Streptococcus mutans*	ACCGTTTAG	12	*smu.150; smu.423; smu.1906*	bacteriocin and mutacin production, peptide quorum sensing	(Xie *et al*., 2010)
ComE	*S. pneumoniae*	(A/T)CA(T/G)TT(C/G)(A/G)G	12	*comCDE; comAB*	competence, peptide quorum sensing	(Ween *et al*., 1999)
FsrA	*Enterococcus faecalis*	(T/A)(T/C)A(A/G)GGA(A/G)	13	*fsrBDC*; *gelE-sprE*	virulence, peptide quorum sensing	(Del Papa & Perego, 2011)
PlnC, PlnD	*Lactobacillus plantarum*	TACGTTAAT	12	*pln* operon	bacteriocin production, peptide quorum sensing	(Risoen *et al*., 2001)
VirR	*Clostridium perfringens*	CCCAGTT(A/C)T(T/G)CAC	8	*pfoA*, *ccp*, *virU*, *virT*, *vrr*	virulence	(Cheung & Rood, 2000)
YehT	*Escherichia coli*	ACC(G/A)CT(C/T)A	13	*yjiY*	unknown	(Chapter 3)
YpdB	*Escherichia coli*	GGCATTTCAT	11	*yhjX*	unknown	(Chapter 4)

* three binding sites

sequence of YehT (Chapter 3) enhanced promoter activity, while, in the case of YpdB (Chapter 4) decreased promoter activity.

Moreover, substitutions of nucleotides adjacent to the DNA-binding sites, whether upstream or downstream, decreased promoter activity. This provides the first evidence that the RR-DNA interaction is stabilized by the intervening spacer as well as the upstream and downstream regulatory sequences. Indeed, such a stabilizing interaction hasbeen shown for the LytTR-like RR FsrA in *E. faecalis* (Del Papa & Perego, 2011). Here, promoter activity was modulated by the sequence-dependent structure of the surrounding DNA. Similarly, binding of the LytTR-like regulators PlnD and PlnC to target promoter DNA was strongly influenced by variations within the spacer sequence (Risøen *et al.*, 2001). This raises the fundamental question of, how DNA bending affects gene regulation. One might speculate, that LytTR-like proteins induce bending of DNA, a process that may play a hitherto underappreciated role in transcriptional regulation (Coulombe, 1999).

In conclusion, the data gathered in the course of this thesis give detailed insights into signal perception and the generation of a cellular response by the YehU/YehT and YpdA/YpdB systems in *Escherichia coli*. In addition, the results help to understand how multiple signals can be externally sensed and integrated to generate an appropriate intracellular response by a complex signaling network.

7.8 Outlook

Of the remaining questions regarding YehU/YehT and YpdA/YpdB signal transduction, some of the most fascinating are the following: What physiological roles do these systems play in the infection processes of pathogenic organisms? What are the ligands that these HKs bind and what are the structural rearrangements that occur in their networks? What are the cellular levels of proteins, and which of their residues are essential for signaling and network formation? What are the spatial and temporal dynamics of complex formation/decomposition and their accessory proteins in vivo? Does network design ensure signaling fidelity? Are additional proteins involved in network formation and signaling, and how did they evolve? Are these systems involved in quorum sensing?

In order to determine whether YehU/YehT and YpdA/YpdB are involved in colonization processes, colonization studies of different pathogenic strains in well-established infection models need to be developed. This could be realized by the systematic generation of deletions in the *E. coli* serotype, O157:H7, and by performing subsequent colonization assays in the mouse model (Njoroge & Sperandio, 2012). Also, to understand the molecular mechanisms of signal perception, it is crucial to identify and characterize all stimuli. Purification of supernatants, followed by liquid chromatography and mass spectrometric analysis could determine the missing ligand for the HK, YehU. Subsequent studies by ITC

(isothermal titration calorimetry), thermophoresis or flow dialysis can be used to determine the affinities of the ligands in both systems (Gerharz *et al.*, 2003, Duurkens *et al.*, 2007, Wienken *et al.*, 2010). In order to determine whether the ratio between the network proteins plays a crucial rule, the signal-dependent protein levels could be analyzed. This could be realized by shifting cells into inducing conditions and comparing the levels of proteins with the aid of specific antibodies and Western blotting. Identification of essential residues in signaling and network formation could be verified by in vivo expression analysis and gel retardation experiments. Therefore, a series of different variants, containing single amino acid substitutions, deletions or insertions within favorable regions, could prove useful in this endeavor. The dynamics of network formation could be analyzed in vivo by fluorescence resonance energy transfer spectroscopy (Gao *et al*., 2008) or by fluorescent hybrid reporter strains (Donovan *et al.*, 2012). The structural arrangement of individual components or temporary complexes within the singaling network could be elucidated by 3D-crystallization, immunogold labeling and electron microscopy (Chen *et al.*, 2002), or cryo-electron tomography (Briegel *et al.*, 2012). Moreover, solved structures will gain further information for ligand binding. Finally, for a complete understanding of LytS/LytTR-like signaling, a transposon mutagenesis screening approach (Boehm *et al.*, 2010) may be considered. This library could give insights into the genes involved in the synthesis and export of unknown quorum sensing molecules, and could also identify new components of the network.

7.9 References for Concluding Discussion

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Supplemental Material – Chapter 2

Supplemental Material for

A comprehensive toolbox for the rapid construction of *lacZ* fusion reporters

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Table S1. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide Sequence (5´-3´)	Description
Plasmid or strain construction		
P lac_rpsL-neo_up	TATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACA GCTGGCCTGGTGATGATGGCGGGATCG	*E. coli LF1 & LF2* construction
lacZ-100bprplsneodown	AACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCC AGCTGGCGATCAGAAGAACTCGTCAAGAAGGCG	*E. coli LF1* construction
lacZ_rpsL-neo_down	CAGTCACGACGTTGTAAAACGACGGCCAGTGAATCCGTAATCATGGT CATTCAGAAGAACTCGTCAAGAAGGCG	*E. coli LF2* construction
lacZ sense	ATGACCATGATTACGGATTCACT	*lacZ* homology arm
lacZ 500bp anti	CGACTGTCCTGGCCGTAACCGACC	*lacZ* homology arm
lacI 583bp sense	GTCTGCGTCTGGCTGGCTGGCATA	*lacI* homology arm
lacI anti	TCACTGCCCGCTTTCCAGTCGGGAA	*lacI* homology arm
LacI_upKdpFABC_sense	ATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGG GCAGTGAGCCTTTTCGGCCTGATCCATCCACACC	*E.coli* LF3 construction
upKdpFABC_flan_lacZ_anti	TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATCCGTAAT CATGGTCATAGTGCACCTCCAGTGGCCTAAAAGTGAT	*E.coli* LF3 construction
PspOMI-RBS-NcoI-lacZ-Fw	ACGT**GGGCCC**AGGAGGACGTCCATGGCTACCATGATTACGGATTCA CTGGC	pBBR1MCS-X-*lacZ* or pNPTS-*lacZ* construction
SpeI-lacZ-Rev	GC**ACTAGT**TTATTTTTGACACCAGACCAACTGGT	pNPTS-*lacZ* construction
KpnI-lacZ-Rev	GC**GGTACC**TTATTTTTGACACCAGACCAACTGGT	pBBR1MCS-X-*lacZ* construction
BamHI-PkdpFABC-Fw	CGA**GGATCC**CCTTTTCGGCCTGATCCATCCA	pBBR1MCS-2,4,5-*kdp-lacZ* or pNPTS-*kdp-lacZ* construction
PspOMI-PkdpFABC-Rev	GCAT**GGGCCC**CTGCATAGTGCACCTCCAGTGGCCTA	pBBR1MCS-2,4,5-*kdp-lacZ*
XbaI-PkdpFABC-Fw	CGA**TCTAGA**CCTTTTCGGCCTGATCCATCCA	pBBR1MCS-3-*kdp-lacZ* construction
NcoI-PkdpFABC-Rev	GGA**CCATGG**AGTGCACCTCCAGTGGCCTA	pNPTS-*kdp-lacZ* construction
Sequencing or controls		
PkdpFABC-chk	GCTGACAGGCGAAACCCTACAG	sequencing and control primer
lacZ 220bp anti	AGCTTTCCGGCACCGCTTCTG	sequencing and control primer
RED-Kan sense	TATCAGGACATAGCGTTGGCTACC	sequencing and control primer
check kan23bpanti	TGCAATCCATCTTGTTCAATCAT	sequencing and control primer

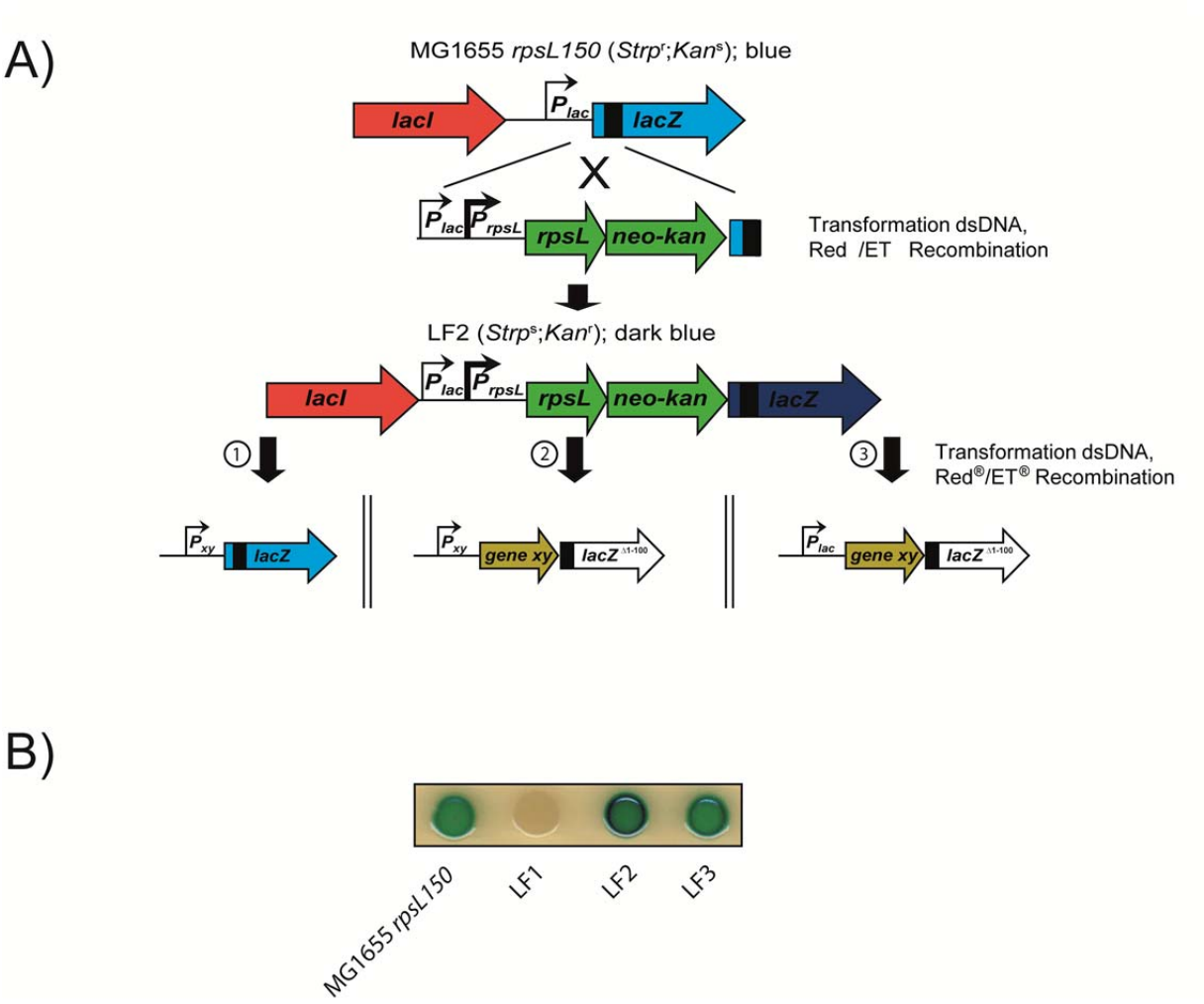

Fig. S1. Schematic depiction of the use of the s*lacZ* tool for construction of reporter fusions in the *E. coli* chromosome. The system is based on Red®/ET®-driven homologous recombination, *rpsL*-based counter-selection and white/blue screening. The starting strain (in this case, *E. coli* MG1655 *rpsL150*) carries a chromosomally encoded streptomycin resistance mutation in *rpsL*. The *rpsL-neo* cassette, flanked by appropriate 50-bp homology arms, is first inserted in the *lac* operon by Red/ET recombination. The resulting strain (LF2) is Kan^r and forms dark-blue colonies on X-Gal plates, because *lacZ* is now expressed from the strong promoter on the *neo-kan* cassette. The additional wild-type allele of *rpsL* makes the strain *Strp*^{s}. In the next step, the *rpsL*-neo cassette is replaced (again with the aid of Red®/ET® recombination) by a double-stranded (ds) DNA fragment comprising the promoter or gene of interest flanked by 500-bp homology arms derived from *lacZ* and *lacI*. Positive clones are *Strp*^{r} and exhibit a blue phenotype on X-Gal plates (due to the recovery of a functional *lacZ*). With this tool, promoter-*lacZ* fusions (1), promoter-gene-*lacZ* fusions (2) or *lac* promoter-gene-*lacZ* fusions can be constructed (3). B) Colonies of *E. coli* MG1655 *rpsL150* and mutants LF1, LF2 and LF3 on LB agar plates containing X-Gal.

Supplemental Material – Chapter 3

Supplemental Material:

First insights into the unexplored two-component system YehU/YehT in *Escherichia coli*

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Table S1. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide Sequence (5´-3´)	Description
Plasmid or strain construction		
yehT XbaI anti	TTTTCTAGATTACAGGCCAATCGCCTCTTT	pBAD24-*yehT*
yehT N-6His EcoRI sense	TTTGAATTCATGCATCATCATCATCATCATAGCAGCGGCCATATCGAAGG TCGTCATATGATTAAAGTCTTAATTGTC	pBAD24-*yehT*
D54E sense	CCGGATGTGCTGTTTCTCGAGATCCAGATGCCGCGCATC	pBAD24-*yehT*-D54E
D54E anti	GATGCGCGGCATCTGGATCTCGAGAAACAGCACATCCGG	pBAD24-*yehT*-D54E
D54N sense	GTGCTGTTTCTCAATATCCAGATGC	pBAD24-*yehT*-D54N
D54N anti	GCATCTGGATATTGAGAAACAGCAC	pBAD24-*yehT*-D54N
kdpE sense	TTTCGCCATATGACAAACGTTCTGATTGT	pBAD24-*kdpE*
kdpE antisense	CTCTCTAGATCAAAGCATAAACCGATAGC	pBAD24-*kdpE*
yjiY EcoRI sense	ATACCGGAATTCATGGATACTAAAAAGATATTCAAGCACA	pBAD24-*yjiY*
yjiY C-6His XbaI anti	TTTTCTAGATTAATGATGATGATGATGATGGCCGCCGCTGCTACGACCTT CGATCATATGGTGGTGCGAAGAGATCTTCACGCCGCCTT	pBAD24-*yjiY*
yehU EcoRI sense	TCGGAATTCATGTACGATTTTAATCTGGTG	pBAD24-*yehU*, pBAD24-*yehUT*
yehU C-6his XbaI	TTTTCTAGATTAATGATGATGATGATGATGATGGCCGCCGCTACGACCTT CGATCATATGTGCCTCGTCC	pBAD24-*yehU*
H382Q sense	GGTGAATCCCCAATTTTTGTTTAAT	pBAD24-*yehU* H382Q
H382Q anti	ATTAAACAAAAATTGGGGATTCACC	pBAD24-*yehU* H382Q
pBAD24 anti	CAAATTCTGTTTTATCAGACCGCTTCTGCG	pBAD24 sequencing
pBAD24 sense	TCGCAACTCTCTACTGTTTCTCCATA	pBAD24 sequencing
rev24	TTCACACAGGAAACAGCTATGACC	pUC19 sequencing, labeling EMSA
uni24	ACGACGTTGTAAAACGACG	pUC19 sequencing
up yjiY 300bp BamHI sense	AATCCGGATCCCGCCGAGTGAATTTTATTCACACTCTGAA	pBBR *yjiY-lux*
up yjiY EcoRI anti	ATACCGGAATTCAGTAAAACCTGGCATGTATTGATTA	pBBR *yjiY-lux*
up yjiY Del CRP BS sense	TAACGCGTGTCGCGTCCGACACGCGTTATCGTCACTTAAACGACGCCTT	pBBR *yjiY*-sub-CRP-BS-*lux*
up yjiY Del CRP BS anti	TAAATTATTCACAATATAACGCGTGTCGCGTCCGACACGCGTTA	pBBR *yjiY*-sub-CRP-BS-*lux*
UP-YehU	ACGCAGGCAATGTATGTTACGCGTTTTAAAGGGAAGTGTGGTTTGCGGG TAATTAACCCTCACTAAAGGGCG	*E. coli* MG2 construction
LOW-YehT	GCATGAGGCCTTCAGGTGTTGATGAGGCAAAAAGCCATTTTAGCAGTCTT TAATACGACTCACTATAGGGCTC	*E. coli* MG2 construction
RED-Kan anti	CGAGACTAGTGAGACGTGCTAC	control primer
RED-Kan sense	TATCAGGACATAGCGTTGGCTACC	control primer
yehT - 50	CGTTACTTAGCATGAGGCCTT	control primer
yehT + 50	TGTGAGCCTGATAGTTACACC	control primer
yehT-rpsL-neo-DOWN	CAAGCATCCCCACCATTTCCAGACCACTGATGCGCGGCATCTGGATATTC AGAAGAACTCGTCAAGAAGG	*E. coli* MG3 construction
yehT-rpsL-neo-up	GAAGGGATCGGCGCGGTGCATAAACTGCGCCCGGATGTGCTGTTTCTCG GCCTGGTGATGATGGCGGGAT	*E. coli* MG3 construction
yehU-rpsL-neo-DOWN	GGGATTCACCTGGGCGTGAAGCAGTTTGATCTCTGACTGGGTGAGCATT CAGAAGAACTCGTCAAGAAGG	*E. coli* MG6 construction
yehU-rpsL-neo-UP	TAAAAATACACTTCGGCTTTTTCTGCCACTGCGCTGAAAGTGGCAGCCGG CCTGGTGATGATGGCGGGAT	*E. coli* MG6 construction
YehTU test anti	GAATAAACAGATGTGTGGTGAGTGT	control primer
YehTU test sense	AAACCCTCTTCGTCTTCTTTACGT	control primer
yehU - 50	GCTCCTGCAAAAATACACGCA	control primer
yehU + 50	CTGCAAGAGTTCAAAGAAAGT	control primer

yehU sense	ATGTACGATTTTAATCTGGTG	control primer
yehU anti	TCATGCCTCGTCCCTCCATGG	control primer
up yjiY	ACCTAGAACGGCTTCGGCCAACTATTAATCAATACATGCCAGGTTTTACT AATTAACCCTCACTAAAGGGCG	*E. coli* MG10 construction
down yjiY	TAGTTCACTCTGATAAGAACAAAGCCCCGCCGAAGCGGGGCTAAACACG GTAATACGACTCACTATAGGGCTC	*E. coli* MG10 construction
yjiY -200	CGTTCGCGGAAGAATTCTTCATA	control primer
up cyaA	GATGTTGGCGGAATCACAGTCATGACGGGTAGCAAATCAGGCGATACGT CAATTAACCCTCACTAAAGGGCG	*E. coli* MG11 construction
down cyaA	GTTTCCGCTAAGATTGCATGCCGGATAAGCCTCGCTTTCCGGCACGTTCA TAATACGACTCACTATAGGGCTC	*E. coli* MG11 construction
cya + 200	GCGCATCTTTCTTTACGGTCAAT	control primer
cya - 200	AGGAGCCGCTGCACCAGGTAT	control primer
up CRP	AGAAAGCTTATAACAGAGGATAACCGCGCATGGTGCTTGGCAAACCGCA AAATTAACCCTCACTAAAGGGCG	*E. coli* MG12 construction
down CRP	GCTACCAGGTAACGCGCCACTCCGACGGGATTAACGAGTGCCGTAAACG ATAATACGACTCACTATAGGGCTC	*E. coli* MG12 construction
CRP + 200	GACACAAAGCGAAAGCTATGCTAAA	control primer
CRP - 200	GTTCCTGCCTGTTGCAATATTGCG	control primer

Northern Blot DNA probes		
alsA sense	ATGGCCACGCCATATATATCG	*alsA* probe
alsA anti	CCAGGGGTGAACGTGGAGAGA	*alsA* probe
arpB sense	ATGAGTCAAAACGATATCATT	*arpB_1 & arpB_2* probe
arpB anti	CTACCATTGAGATTTACTGTT	*arpB_1 & arpB_2* probe
cspI sense	ATGTCTAACAAAATGACTGGT	*cspI* probe
cspI anti	TCAAAGCGCCACTACATGAAC	*cspI* probe
cysB sense	ATGAAATTACAACAACTTCGC	*cysB* probe
cysB anti	TTATTTTTCCGGCAGTTTTAT	*cysB* probe
evgA anti	TTAGCCGATTTTGTTACGTTG	*evgAS* probe
evgA sense	ATGAACGCAATAATTATTGAT	*evgAS* probe
fimB sense	ATGAAGAATAAGGCTGATAAC	*fimB* probe
fimB anti	CTATAAAACAGCGTGACGCTG	*fimB* probe
fimE sense	GTGAGTAAACGTCGTTATCTT	*fimE* probe
fimE anti	TCAAACCTCTTCTCTTTTTAA	*fimE* probe
gadB sense	ATGGATAAGAAGCAAGTAACG	*gadB* probe
gadB anti	GGCGCAGGAATTCATAGTACT	*gadB* probe
ivbL sense	ATGACTACTTCCATGCTCAAC	*ivbL* probe
ivbL anti	CTACGGCGCATTGCCGACGAC	*ivbL* probe
kdsB sense	ATGAGTTTTGTGGTCATTATT	*kdsB* probe
kdsB anti	TTAGCGCATTTCAGCGCGAAC	*kdsB* probe
nlpA sense	ATGAAACTGACAACACATCAT	*nlpA* probe
nlpA anti	TTACCAGCCAGGCACCGCGCC	*nlpA* probe
nupA sense	ATGAAAAAAACATTACTGGCA	*nupA* probe
nupA anti	TCAGAAGTTGTAACCTACTAC	*nupA* probe
ompT sense	ATGCGGGCGAAACTTCTGGGA	*ompT* probe
ompT anti	TTAAAATGTGTACTTAAGACC	*ompT* probe
rpoD sense	ATGGAGCAAAACCCGCAGTCAC	*rpoD* probe
rpoD anti	AATCGTCCAGGAAGCTACGCAGC	*rpoD* probe
yadC sense	ATGAAGACTATCTTCAGGTAC	*yadC* probe
yadC anti	GTACGCCAACGCCTTTGGCGG	*yadC* probe
yahN sense	ATGATGCAGTTAGTTCACTTA	*yahN* probe
yahN anti	TCACCGCTGCGTCACCCCTTC	*yahN* probe
ybbC sense	ATGAAATATAGTTCAATATTT	*ybbC* probe
ybbC anti	TTATTTACTATGTAGGAAATT	*ybbC* probe
ybcK sense	ATGAAAAAAGCCATAGCATAT	*ybcK* probe

ybcK anti	TCATCGGACAAACATAATAGC	*ybcK* probe
yebK sense	ATGAATATGCTGGAAAAAATC	*yebK* probe
yebK anti	TTAGCGATCGTCACTTAAATT	*yebK* probe
yeiL sense	ATGAGTGAATCCGCGTTTAAG	*yeiL* probe
yeiL anti	TTACTGCATCATCCCGGAGAA	*yeiL* probe
yfcV sense	ATGAGTAAGTTTGTTAAAACA	*yfcV* probe
yfcV anti	TTACAGGTAAGTAATCTGGAA	*yfcV* probe
yfiL sense	ATGATGAAAAAGTTTATCGCC	*yfiL* probe
yfiL anti	TTAATTTAATCGTATTGTGCT	*yfiL* probe
yhjX sense	ATGACACCTTCAAATTATCAG	*yhjX* probe
yhjX anti	CAAAGAACTCACTGACCAGTG	*yhjX* probe
yibG sense	ATGAAAGCATGCTTGTTACTA	*yibG* probe
yibG anti	TTACGGATTCTCCTTATTCTT	*yibG* probe
yigF sense	ATGAGTAAGGAATATATGAAC	*yigF* probe
yigF anti	TCAAAGGCTCCAGGTATTTAA	*yigF* probe
yjiY sense	ATGGATACTAAAAAGATATTC	*yjiY* probe
yjiY anti	TGATGAACAGGAACGGGAACA	*yjiY* probe
ylbH sense	ATGAGCGGAAAACCAGCGGCG	*ylbH* probe
ylbH anti	TGCTGCGGTCATATACCGCCA	*ylbH* probe
yobA sense	ATGGCTTCAACTGCACGCTCC	*yobA-yebZY* probe
yobA anti	TTATTTCACGCTAAAGGTGTA	*yobA-yebZY* probe
ypjB sense	ATGGAATCACGTAATTCATAT	*ypjB* probe
ypjB anti	TCATTGAAAACTGCTTTTAGA	*ypjB* probe
ytfI sense	ATGTTACCCAGGATCAGACAC	*ytfI* probe
ytfI anti	TTAAGTCTCTGAGTTCTTACG	*ytfI* probe

5´RACE

5PR RNA Adapter*	GAUAUGCGCGAAUUCCUGUAGAACGAACACUAGAAGAAA	(1)
5´R Adapter Primer III	TGTAGAACGAACACTAGAAGAA	5´RACE Adapter
5PR Primer II	GCGCGAATTCCTGTAGA	5´RACE
cspI-c-anti	CCGTTCTCAATACCAAATTCAACT	*cspI* 5´RACE
cspI-cII-anti	CACATCTTTGCTGCCATCTT	*cspI* 5´RACE
cspI-c-III	CCTTTTTCAGGGTTAAACCATT	*cspI* 5´RACE
yhjX-c-anti	AAACAGGCTCCAGGTATAAACC	*yhjX* 5´RACE
yhjX-c-anti II	CGCAACAGAAGACGAAATTG	*yhjX* 5´RACE
yjiY c anti II	GTTCAGACCGTCGTTGTTAATAA	*yjiY* 5´RACE
yjiY-c-anti	CCGACGTAATGCAACTACCG	*yjiY* 5´RACE
yjiY-cII-anti	GTTCAGACCGTCGTTGTTAATAA	*yjiY* 5´RACE
ypjB-c-anti	TCTATGGGGTGTTCCTTT	*ypjB* 5´RACE
ypjB-c-anti II	GCTCTTGGTAAAAACTTATAGCAAC	*ypjB* 5´RACE

EMSA/footprint

6-FAM uni24	[6-FAM]ACGACGTTGTAAAACGACGGCCAG	EMSA labeling DNA-fragments
cspI 1 anti	TTTTTTGGATCCTGAGAAATGGACAAACAC	pUC19 $P_{cspI\ -150/+250}$
cspI 4 sense	TTTTTTGAATTCGCCAAAATTCCTGAAATC	pUC19 $P_{cspI\ -150/+250}$
yhjX 5PR 1 anti	TTTTTTGGATCCTAAACAGGCTCCAGGTATAA	pUC19 $P_{yhjX\ -173/+137}$
yhjX 5PR 3 sense	TTTTTTGAATTCTAACAATAGTTGTGGCGATAGTGG	pUC19 $P_{yhjX\ -173/+137}$
yjiY YehT bs	TTTTTTGAATTCCCTTTGCCGCTCAACCGCAAAACTGACCGCTTACATCC CTAAAATAACCACTCAGTTAGGATCCTTTTTT	pUC19 $P_{yjiY\ P}$
yjiY YehT bs anti	AAAAAAGGATCCTAACTGAGTGGTTATTTTAGGGATGTAAGCGGTCAGTT TTGCGGTTGAGCGGCAAAGGGAATTCAAAAAA	pUC19 $P_{yjiY\ P}$
yjiY YehT bs mut 1	TTTTTTGAATTCCCTTTGAATAGACACCGCAAAACTGACCGCTTACATCCC TAAAATAACCACTCAGTTAGGATCCTTTTTT	pUC19 $P_{yjiY\ MM3}$
yjiY YehT bs mut 1 anti	AAAAAAGGATCCTAACTGAGTGGTTATTTTAGGGATGTAAGCGGTCAGTT TTGCGGTGTCTATTCAAAGGGAATTCAAAAAA	pUC19 $P_{yjiY\ MM3}$

yjiY YehT bs mut 2	TTTTTTGAATTCCCTTTGAATAGACACCGCAAAACTGAAATAGGCCATCCCTAAAATAACCACTCAGTTAGGATCCTTTTTT	pUC19 $P_{yjiY\ MM23}$
yjiY YehT bs mut 2 anti	AAAAAAGGATCCTAACTGAGTGGTTATTTTAGGGATGGCCTATTTCAGTTTTGCGGTGTCTATTCAAAGGGAATTCAAAAAA	pUC19 $P_{yjiY\ MM23}$
yjiY YehT bs mut 3	TTTTTTGAATTCCCTTTGAATAGACACCGCAAAACTGAAATAGGCCATCCCTAAAATAAAAGAGACGTTAGGATCCTTTTTT	pUC19 $P_{yjiY\ MM123}$
yjiY YehT bs mut 3 anti	AAAAAAGGATCCTAACGTCTCTTTTATTTTAGGGATGGCCTATTTCAGTTTTGCGGTGTCTATTCAAAGGGAATTCAAAAAA	pUC19 $P_{yjiY\ MM123}$
yjiY YehT bs spacer mut	TTTTTTGAATTCCCTTTGCCGCTCACAATACCCCAGTCCCGCTTAACGAAAGCCCCGCCCCACTCAGTTAGGATCCTTTTTT	pUC19 $P_{yjiY\ MS}$
yjiY YehT bs spacer mut anti	AAAAAAGGATCCTAACTGAGTGGGGCGGGGCTTTCGTTAAGCGGGACTGGGGTATTGTGAGCGGCAAAGGGAATTCAAAAAA	pUC19 $P_{yjiY\ MS}$
yjiY-5P-1 anti	TTTTTTGGATCCAGTAAAACCTGGCATGTA	pUC19 $P_{yjiY\ -212/+88}$, pUC19 $P_{yjiY\ -12/+88}$, pRS415 $P_{yjiY\ -212/+88}$ + derivates
yjiY-5P-1 sense	TTTTTTGAATTCCAACATCACTACAGGATAG	pUC19 $P_{yjiY\ -12/+88}$
yjiY-5P-2 anti	TTTTTTGGATCCCCAGAGTTACGCGCGCGT	pUC19 $P_{yjiY\ -112/-13}$
yjiY-5P-2 sense	TTTTTTGAATTCCTTAAACGACGCCTTTGCCGC	pUC19 $P_{yjiY\ -112/-13}$
yjiY-5P-3 anti	TTTTTTGGATCCTGACGATAAATATGTGAT	pUC19 $P_{yjiY\ -212/-113}$
yjiY-5P-3 sense	TTTTTTGAATTCCGCCGAGTGAATTTTATTCA	pUC19 $P_{yjiY\ -212/+88}$, pUC19 $P_{yjiY\ -212/-113}$ pRS415 $P_{yjiY\ -212/+88}$ + derivates
ypjB 5PR 1 anti	TTTTTTGGATCCGAACTAACTAACTCGTGATTA	pUC19 $P_{ypjB\ -170/+130}$
ypjB 5PR 3 sense	TTTTTTGAATTCATTTAACGCTAGCGCAGTTTT	pUC19 $P_{ypjB\ -170/+130}$

In vivo reporter

2STPCR_ms_sense	CAATACCCCAGTCCCGCTTAACGAAAGCCCCGCC	pRS415 $P_{yjiY\ MS}$
2STPCR_ms_antis	GGCGGGGCTTTCGTTAAGCGGGACTGGGGTATTG	pRS415 $P_{yjiY\ MS}$
2STPCR_m123_sense	AATAGACACCGCAAAACTGAAATAGGCCATCCCTAAAATAAAAGAGAC	pRS415 $P_{yjiY\ M123}$
2STPCR_m123_anti	GTCTCTTTTATTTTAGGGATGGCCTATTTCAGTTTTGCGGTGTCTATT	pRS415 $P_{yjiY\ M123}$
2STPCR_m23_sense	AATAGACACCGCAAAACTGAAATAGGC	pRS415 $P_{yjiY\ M23}$
2STPCR_m23_anti	GCCTATTTCAGTTTTGCGGTGTCTATT	pRS415 $P_{yjiY\ M23}$
2STPCR_m3_sense	AATAGACACCGCAAAACTGAC	pRS415 $P_{yjiY\ M3}$
2STPCR_m3_anti	GTCAGTTTTGCGGTGTCTATT	pRS415 $P_{yjiY\ M3}$
yjiY spacer -1 sense	CATCCCGAAAATAACCACTCAG	pRS415 $P_{yjiY\ SC1}$
yjiY spacer -1 antisense	CTGAGTGGTTATTTTCGGGATG	pRS415 $P_{yjiY\ SC1}$
yjiY spacer -3 sense	CATCCAGCAAATAACCACTCAG	pRS415 $P_{yjiY\ SC3}$
yjiY spacer -3 antisense	CTGAGTGGTTATTTGCTGGATG	pRS415 $P_{yjiY\ SC3}$
yjiY spacer -7 sense	CATAAAGCCCATAACCACTCAG	pRS415 $P_{yjiY\ SC7}$
yjiY spacer -7 antisense	CTGAGTGGTTATGGGCTTTATG	pRS415 $P_{yjiY\ SC7}$
yjiY spacer -13 sense	ACGAAAGCCCCGCACCACTCAGTTATT	pRS415 $P_{yjiY\ SC13}$
yjiY spacer -13 antisense	AATAACTGAGTGGTGCGGGGCTTTCGT	pRS415 $P_{yjiY\ SC13}$

* RNA-Oligonucleotide

Table S2. Genes most affected by the overexpression of *yehT* or *kdpE.*

gene[a]	b-number[a]	rF (YehT)[b]	rF (KdpE)[b]	log2 Ratio[b]	p[c]	function[a]	transcriptional regulation[d]
yjiY	b4354	9,200	50	7.6	$\leq 10^{-3}$	predicted inner membrane protein	YehT ↑
evgA	b2369	3,000	70	5.4	$\leq 10^{-3}$	DNA-binding transcriptional activator EvgA	KdpE ↓
yobA	b1841	3,200	170	4.2	$\leq 10^{-3}$	hypothetical protein	KdpE ↓
nlpA	b3661	1,300	70	4.2	0.003	cytoplasmic membrane lipoprotein-28	KdpE ↓
evgS	b2370	700	40	4.2	$\leq 10^{-3}$	hybrid HK in two-component regulatory system with EvgA	KdpE ↓
yebZ	b1840	2,100	150	3.8	$\leq 10^{-3}$	putative resistance protein	KdpE ↓
nupA	b0411	1,700	140	3.6	0.001	nucleoside channel	KdpE ↓
ivbL	b3672	3,700	330	3.5	$\leq 10^{-3}$	*ilvB* operon leader peptide	KdpE ↓
ompT	b0565	8,200	770	3.5	$\leq 10^{-3}$	outer membrane protease	KdpE ↓
yahN	b0328	530	60	3.3	$\leq 10^{-3}$	putative cytochrome subunit of dehydrogenase	KdpE ↓
yebK	b1853	1,880	210	3.2	$\leq 10^{-3}$	DNA-binding transcriptional regulator HexR	KdpE ↓
cysB	b1275	1,890	230	3.0	$\leq 10^{-3}$	transcriptional regulator CysB	KdpE ↓
kdsB	b0918	2,390	300	3.0	$\leq 10^{-3}$	3-deoxy-manno-octulosonate cytidylyltransferase	KdpE ↓
cspI	b1552	300	2,480	-3.0	0.01	cold shock-like protein CspI	YehT ↓
fimB	b4312	950	7,810	-3.1	0.001	tyrosine recombinase/inversion of on/off regulator of *fimA*	KdpE ↑
yadC	b0135	50	510	-3.2	$\leq 10^{-3}$	predicted fimbrial-like adhesin protein	KdpE ↑
yigF	b3817	50	510	-3.3	$\leq 10^{-3}$	conserved inner membrane protein	KdpE ↑
gadB	b1493	750	7,340	-3.5	$\leq 10^{-3}$	glutamate decarboxylase B. PLP-dependent	KdpE ↑
arpB_2	b1721	40	420	-3.9	0.001	hypothetical protein	KdpE ↑
ybbC	b0498	40	420	-4.2	0.001	predicted protein	KdpE ↑
alsA	b4087	180	3,110	-4.4	$\leq 10^{-3}$	fused D-allose transporter subunits of ABC superfamily: ATP-binding components	KdpE ↑
yibG	b3596	30	530	-4.5	$\leq 10^{-3}$	hypothetical protein	KdpE ↑
yeiL	b2163	30	590	-4.5	$\leq 10^{-3}$	DNA-binding transcriptional activator	KdpE ↑
yfcV	b2339	50	1,020	-4.6	0.003	predicted fimbrial-like adhesin protein	KdpE ↑
ytfI	b4215	10	180	-4.8	0.008	hypothetical protein	KdpE ↑
yfiL	b2602	30	830	-5.0	0.001	hypothetical protein	KdpE ↑
ylbH	b0499	180	5,480	-5.1	0.001	protein in rhs loci	KdpE ↑
ybcK	b0544	70	2,540	-5.2	$\leq 10^{-3}$	DLP12 prophage; predicted recombinase	KdpE ↑
fimE	b4313	10	400	-5.3	0.003	tyrosine recombinase	KdpE ↑
yhjX	b3547	80	3,120	-5.3	$\leq 10^{-3}$	uncharacterized member of the major facilitator superfamily (MFS) of transporters	YehT ↓
arpB_1	b1720	200	7,980	-6.9	0	hypothetical protein	KdpE ↑
ypjB	b2649	20	1,790	-8.3	$\leq 10^{-3}$	hypothetical protein	YehT ↓

[a] Gene names/b-numbers and gene product function are adopted from http://www.ecocyc.org (3) and the Affymetrix Expression Analysis Sequence Information Database (2).
[b] Log_2 ratio of transcript levels for the *yehT*- and *kdpE*-overexpression strains. Log_2 was calculated from the ratio of the mean fluorescence intensity of the respective transcript in the *yehT*-overexpressing [rF (YehT)] to that measured in the *kdpE* overexpression [rF (KdpE)] strain. A negative (positive) value denotes a decrease (increase) in transcription level upon YehT overproduction in comparison to that seen in the KdpE overproduction strain.
[c] p significance (t-test) of single rF values.
[d] Effect of YehT-D54E or KdpE overproduction on the transcript levels of the respective genes in *E. coli* MG2 compared to control cells (*E. coli* MG2, pBAD24), as determined by Northern blot analysis. YehT-dependent, respectively KdpE-dependent, induction (YehT ↑, KdpE ↑) or repression (YehT ↓, KdpE ↓) of the gene is indicated (see Figure 3.2A).

Table S3. Internal control genes affected by the overexpression of *yehT* or *kdpE.*

gene[a]	b-number[a]	rF (YehT)[b]	rF (KdpE)[b]	log2 Ratio[b]	p[c]	function[a]
yehT	b2125	11,240	2,060	2.5	≤ 0,01	two-component system response regulator
yehU	b2126	640	1,090	-0.8	0,065	two-component system histidine kinase
kdpE	b0694	930	17,380	-4.2	0,013	two-component system response regulator
kdpD	b0695	570	7,370	-3.7	≤ 0,001	two-component system histidine kinase
kdpF	b4513	50	5,020	-6.6	≤ 0,001	subunit of P-Type ATPase potassium ion transporter
kdpA	b0698	600	10,200	-4.1	≤ 0,001	subunit of P-Type ATPase potassium ion transporter
kdpB	b0697	430	7,570	-4.2	≤ 0,001	subunit of P-Type ATPase potassium ion transporter
kdpC	b0696	630	10,130	-4.0	≤ 0,001	subunit of P-Type ATPase potassium ion transporter

[a] Gene names/b-numbers and gene product function are adopted from http://www.ecocyc.org (3) and the Affymetrix Expression Analysis Sequence Information Database (2).
[b] Log_2 ratio of transcript levels for the *yehT*- and *kdpE*-overexpression strains. Log_2 was calculated from the ratio of the mean fluorescence intensity of the respective transcript in the *yehT*-overexpressing [rF (YehT)] to that measured in the *kdpE* overexpression [rF (KdpE)] strain. A negative (positive) value denotes a decrease (increase) in transcription level upon YehT overproduction in comparison to that seen in the KdpE overproduction strain.
[c] p significance (t-test) of single rF values.
[d] Effect of YehT-D54E or KdpE overproduction on the transcript levels of the respective genes in *E. coli* MG2 compared to control cells (*E. coli* MG2, pBAD24), as determined by Northern blot analysis. YehT-dependent, respectively KdpE-dependent, induction (YehT ↑, KdpE ↑) or repression (YehT ↓, KdpE ↓) of the gene is indicated (see Figure 3.2A).

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Supplemental Material – Chapter 4

Supplemental Material:

Identification of a target gene and activating stimulus for the YpdA/YpdB histidine kinase/response regulator system in *Escherichia coli*

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Running title: The YpdA/YpdB-system in *E. coli*

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Table S1. Plasmids used in this study.

Plasmid	Description	Reference or source
pRed/ET	λ-RED recombinase in pBAD24; Amp^r	Gene Bridges
pCP20	FLP-recombinase, λcI 857^+, λpR Rep^{ts}; Amp^r, Cm^r	(1)
pBAD33-Cm	Arabinose-inducible P_{BAD} promoter, pBR322 ori; Kan^r	(2)
pBAD33-*ypdB*	*6his-ypdB* cloned in the AflII and XbaI sites of pBAD33-Cm; Cm^r	This work
pBAD24	Arabinose-inducible P_{BAD} promoter, pBR322 ori; Amp^r	(2)
pBAD24-*ypdB*	*6his-ypdB* cloned in the EcoRI and XbaI sites of pBAD24; Amp^r	This work
pBAD24-*ypdB* D53E	*ypdB* D54E cloned in the NdeI and XbaI sites of pBAD24-*ypdB*; Amp^r	This work
pBAD24-*ypdB* D53N	*ypdB* D54N cloned in the NdeI and XbaI sites of pBAD24-*ypdB*; Amp^r	This work
pBAD24-*yehS*	*yehS* cloned in the NdeI and XbaI sites of pBAD24-*kdpE*; Amp^r	This work
pBAD24-*ypdA*	*ypdA* cloned in the EcoRI and XbaI sites of pBAD24; Amp^r	This work
pBAD24-*ypdA* H371Q	*ypdA* H371Q cloned in the EcoRI and XbaI sites of pBAD24; Amp^r	This work
pBAD24-*yhjX*	*yhjX-6his* cloned in the EcoRI and XbaI sites of pBAD24; Amp^r	This work
pBAD24-*ypdAB*	*ypdAB* cloned in the EcoRI and XbaI sites of pBAD24; Amp^r	This work
pBAD24-*ypdABC*	*ypdABC* cloned in the EcoRI and XbaI sites of pBAD24; Amp^r	This work
pUC19	IPTG-inducible P_{Lac} promoter, pMB1 ori, Amp^r	(3)
pUC19 $P_{yjiY\ -212/+88}$	$P_{yjiY\ -212/+88}$ cloned in the EcoRI and BamHI sites of pUC19; Amp^r	(4)
pUC19 $P_{yhjX\ -264/+36}$	$P_{yhjX\ -264/+36}$ cloned in the EcoRI and BamHI sites of pUC19; Amp^r	This work
pUC19 $P_{yhjX\ -264/-165}$	$P_{yhjX\ -264/-165}$ cloned in the EcoRI and BamHI sites of pUC19; Amp^r	This work
pUC19 $P_{yhjX\ -164/-65}$	$P_{yhjX\ -164/-65}$ cloned in the EcoRI and BamHI sites of pUC19; Amp^r	This work
pUC19 $P_{yhjX\ -64/+36}$	$P_{yhjX\ -64/+36}$ cloned in the EcoRI and BamHI sites of pUC19; Amp^r	This work
pRS415	Operon fusion vector	(5)
pRS415 $P_{yhjX\ -264/+36}$	$P_{yhjX\ -264/+36}$ cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ up\_rplmt}$	$P_{yhjX\ up\_rplmt}$ (replacement of 15 bp upstream of M1) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ M1}$	$P_{yhjX\ M1}$ (replacement of M1) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ spacer}$	$P_{yhjX\ spacer}$ (replacement of spacer) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ M2}$	$P_{yhjX\ M2}$ (replacement of M2) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ down\text{-}rplmt}$	$P_{yhjX\ down\_rplmt}$ (replacement of 15 bp downstream of M2) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ M2S1}$	$P_{yhjX\ M2S1}$(replacement of bp 1 and 10 in M2) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ M2S2}$	$P_{yhjX\ M2S2}$(replacement of bp 1,2,9 and 10 in M2) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pRS415 $P_{yhjX\ M2S3}$	$P_{yhjX\ M2S3}$ (replacement of bp 1,2,3,8,9 and 10 in M2) cloned in the EcoRI and BamHI sites of pRS415; Amp^r	This work
pBBR1-MCS5-TT-RBS-*lux*	*luxCDABE* and terminators lambda *T0 rrnB1 T1* cloned into pBBR1-MCS5 for plasmid-based transcriptional fusions; Gm^r	(6)
pBBR *yhjX-lux*	$P_{yhjX\ -264/+36}$ cloned in the BamHI and EcoRI sites of pBBR1-MCS5-TT-RBS-*lux*; Gm^r	This work

Table S2. Oligonucleotides used in this study

Oligonucleotide	#	Oligonucleotide Sequence (5´-3´)	Description
Plasmid or strain construction			
YpdB NdeI sense		AACATATGGTGAAAGTCATCATTGTTGAA	pBAD24-*ypdB*
YpdB XbaI antisense		CCTCTAGATTAAAGATGCATTAACTGGCG	pBAD24-*ypdB,* pBAD24-*ypdAB*
ypdB B53E sense		GCCATTTTTCTGGAAATCAATATTCCG	pBAD24-*ypdB*-D53E
ypdB B53E antisense		CGGAATATTGATTTCCAGAAA AATGGC	pBAD24-*ypdB*-D53E
ypdB D53N sense		ATAACCGCGTCGACGCCATTT TTCTGAATATCA ATATTCCGTCGCTGG ATGGCG T	pBAD24-*ypdB*-D53N
ypdB D53N anti		ACGCCATCCAGCGACGGAATATTGATATTCAGAAAAATGGCGTCGAC GCGGTTAT	pBAD24-*ypdB*-D53N
yehS NdeI sense		ATGCGCCATATGCTAAGTAACGATATTCTGC	pBAD24-*yehS*
yehS XbaI antisense		CTCTCTAGATTAGCCTTTTTTCACATGCT	pBAD24-*yehS*
yhjX EcoRI sense		CAGGAGGAATTCATGACACCTTCAAATTATCAGC	pBAD24-*yhjX*
yhjX NdeI anti		GGAATTCCATATGAAGGGAGCCATGCGCCTCACGCAAC	pBAD24-*yhjX*
YpdA EcoRI sense		CCGAATTCGTGCACGAAATATTCAACATG	pBAD24-*ypdA,* pBAD24-*ypdAB,* pBAD24-*ypdABC,* pBAD24-*ypdA* H371Q
YpdA NdeI antisense		AACATATGAAGCAATAACGTAGCCTGTGA	pBAD24-*ypdA,* pBAD24-*ypdA* H371Q
YpdC XbaI antisense		CCTCTAGATTAGCCCTGAAAACGGGCGCT	pBAD24-*ypdABC*
ypdA H371Q sense		TCGCGCCCTGCAAAGCAAAATTAATCCCCAGTTTCTGTTTAACGCTCT GAACGCTATTTCA	pBAD24-*ypdA* H371Q
ypdA H371Q anti		TGAAATAGCGTTCAGAGCGTTAAACAGAAACTGGGGATTAATTTTGCT TTGCAGGGCGCGA	pBAD24-*ypdA* H371Q
pBAD24 anti		CAAATTCTGTTTTATCAGACCGCTTCTGCG	pBAD24 sequencing
pBAD24 sense		TCGCAACTCTCTACTGTTTCTCCATA	pBAD24 sequencing
rev24		TTCACACAGGAAACAGCTATGACC	pUC19 sequencing, labeling EMSA
uni24		ACGACGTTGTAAAACGACG	pUC19 sequencing
up yhjX 300bp BamHI sense		AATCCGGATCCCTAACTCAGGCAGAAAATACCA	pBBR *yhjX-lux*
up yhjX EcoRI anti		ATACCGGAATTCGGCAGTATTCCTGCAGTAATAAAAAG	pBBR *yhjX-lux*
Up YpdA		AGCCTTCAGGTTACCTATCATAGAGGTTTAATCCTTATTCAGAGTCAC CCAATTAACCCTCACTAAAGGGCGG	*E. coli* MG20 construction
Low YpdC		GATGCACAAAGTATCCTGACGCTGCTGGAAACAGAATTAACCTTCTGA CGTAATACGACTCACTATAGGGCTCG	*E. coli* MG20 construction
YpdBC-rpsL-neo-up		AACAGGAACTGAGCTGGCTAATTAAAGAGCACAGCCAGATGGAGATT GTCGGCACCTTTGGGCCTGGTGATGATGGCGGGATCG	*E. coli* MG21 construction
YpdBC-rpsL-neo-down		GCAAGATGCACAAAGTATCCTGACGCTGCTGGAAACAGAATTAACCT TCTGACGTCAGAAGAACTCGTCAAGAAGGCG	*E. coli* MG21 construction
RED-Kan anti		CGAGACTAGTGAGACGTGCTAC	control primer
RED-Kan sense		TATCAGGACATAGCGTTGGCTACC	control primer
ypdB sense		CGTTACTTAGCATGAGGCCTT	control primer
ypdB +84 sense		TGTGAGCCTGATAGTTACACC	control primer
ypdA +350 s		CCGGACCGTCCGAGCGACGCT	control primer
ypdA +50 s		AGCCTTCAGGTTACCTATCAT	control primer
ypdC + 50 a		GATGCACAAAGTATCCTGACG	control primer
ypdC + 350 a		CGCACTGAACATCCGTTTGAG	control primer
down-ypdB-rpsl - D53		CACGCGGTGATGAACACAATAAACGGTTTATGGGCGAACTGGCTGAT GTTTCAGAAGAACTCGTCAAGAAGGCG	*E. coli* MG 24 / MG 25 construction
up ypdA rpsl neo		AATGCTTATCTGCCTGTTCTTTCTCATCCGTATCCGCCTGTTTCGCGA ACGGCCTGGTGATGATGGCGGGATCG	*E. coli* MG23 construction

down ypdA rspl neo	AATGTAAAACGCAATTTCCGTCCCCGGCTCCAGGCGGCGGATATGCA GCCTCAGAAGAACTCGTCAAGAAGGCG	control primer
ypdA sense	GTGCACGAAATATTCAACATG	control primer
ypdA anti	TCAAAGCAATAACGTAGCCT	control primer
YpdA+up50bp sense	AGCCTTCAGGTTACCTATCATAGAGGTTTAATCCTTATTCAGAGTCAC CCGTGCACGAAATATTCAACATG	*E. coli* MG 23 construction
YpdA-down50bp anti	TGCCAGGAATTCGTCTTCAACAATGATGACTTTCACAATATCACTCCG GCTCAAAGCAATAACGTAGCCTGT	*E. coli* MG 23 construction
YpdB+up50bp sense	ACCCCAGTCGCCTCACAGGCTACGTTATTGCTTTGAGCCGGAGTGAT ATTGTGAAAGTCATCATTGTTGAAGA	*E. coli* MG 24 / MG 25 construction
YpdB-down50bp anti	AAAAATTGTTGATCGGCGGGCAAGCCTGGTGCTTTCATGAAAGTTCC CGATTAAAGATGCATTAACTGGCGAAAT	*E. coli* MG 24 / MG 25 construction
up yhjX	TATGGTTGTCGGCAGAGATTTTTCCTTTTTATTACTGCAGGAATACTG CCAATTAACCCTCACTAAAGGGCG	*E. coli* MG26 construction
down yhjX	ATGCGTTTGATGCACACGGAAGCTGAAGCCCAGTAGCTCGCGGCTG AGCATAATACGACTCACTATAGGGCTC	*E. coli* MG26 construction
yhjX-200	GCAAAGGGAAAAAGTGTGGGGA	control primer

Northern Blot DNA probes		
cpxP anti	CTACTGGGAACGTGAGTTGCT	*cpxP* probe
cpxP sense	ATGCGCATAGTTACCGCTGCC	*cpxP* probe
entC anti	TTAATGCAATCCAAAAACGTT	*entC* probe
entC sense	ATGGATACGTCACTGGCTGAG	*entC* probe
entE anti	TGCCAAACACCTGCTGCAACT	*entE* probe
entE sense	ATGAGCATTCCATTCACCCGC	*entE* probe
fecA anti	GCAGGCTGTTGAAGGTGTGCA	*fecA* probe
fecA sense	ATGACGCCGTTACGCGTTTTT	*fecA* probe
fecB anti	TCATTTCACAACGGTAAGCGG	*fecB* probe
fecB sense	ATGTTGGCATTTATCCGTTTT	*fecB* probe
fhuA anti	GCAGGTTCTGACGCACAGTAA	*fhuA*probe
fhuA sense	ATGGCGCGTTCCAAAACTGCT	*fhuA*probe
fhuF anti	TCATTTCAGCGTACAATCGCC	*fhuF* probe
fhuF sense	ATGGCCTATCGTTCCGCACCG	*fhuF* probe
guaC anti	TTACAGGTTGTTGAAGATGCG	*guaC* probe
guaC sense	ATGCGTATTGAAGAAGATCTG	*guaC* probe
iraP anti	TTACTGACGAGGATGCTTCAA	*iraP* probe
iraP sense	ATGAAAAATCTCATTGCTGAG	*iraP* probe
rpoD anti	AATCGTCCAGGAAGCTACGCAGC	*rpoD* probe
rpoD sense	ATGGAGCAAAACCCGCAGTCAC	*rpoD* probe
yahM anti	CTACGTAATCAACCTGATTTG	*yahM* probe
yahM sense	ATGGCGGTCCAACTTTTCAAA	*yahM* probe
yehS anti	TTAGCCTTTTTTCACATGCTG	*yehS* probe
yehS sense	ATGCTAAGTAACGATATTCTG	*yehS* probe
ygbK anti	TTACCCACGGCACGCCGGGGAAAT	*ygbK* probe
ygbK sense	ATGATCAAGATTGGCGTTATC	*ygbK* probe
ygbL anti	TTAACTCCTTAATTCCGCAAT	*ygbL* probe
ygbL sense	ATGAGCGATTTCGCAAAAGTA	*ygbL* probe
yhjX anti	CAAAGAACTCACTGACCAGTG	*yhjX* probe
yhjX sense	ATGACACCTTCAAATTATCAG	*yhjX* probe
yjiY anti	TGATGAACAGGAACGGGAACA	*yjiY* probe
yjiY sense	ATGGATACTAAAAAGATATTC	*yjiY* probe
ynjH anti	TTATGGCTTTACGCGCCGCCA	*ynjH* probe
ynjH sense	GTGAGTCGAGCATTGTTCGCC	*ynjH* probe
ypdB anti	TTAAAGATGCATTAACTGGCG	*ypdB* probe
ypdB sense	GTGAAAGTCATCATTGTTGAA	*ypdB* probe

EMSA/ footprint		
6-FAM uni24	[6-FAM]ACGACGTTGTAAAACGACGGCCAG	EMSA labeling DNA-fragments
yhjX 1 sense	TTGAATTCTTCTGATGGCATTTCATG	pUC19 $P_{yhjX\ -64/+36}$
yhjX 1 anti	TTGGATCCGGCAGTATTCCTGCAGTA	pUC19 $P_{yhjX\ -264/+36}$, pRS415 $P_{yhjX\ -264/+36}$ + derivates
yhjX 2 sense	TTGAATTCTAACAATAGTTGTGGCGA	pUC19 $P_{yhjX\ -164/-65}$
yhjX 2 anti	TTGGATCCCGGAATGAAATGCCTTAG	pUC19 $P_{yhjX\ -164/-65}$
yhjX 3 sense	TTGAATTCCTAACTCAGGCAGAAAAT	pUC19 $P_{yhjX\ -264/-165}$, pUC19 $P_{yhjX\ -264/+36}$
yhjX 3 anti	TTGGATCC TTTAATGGTTTCAATTGT	pUC19 $P_{yhjX\ -264/-165}$
yjiY-5P-1 anti	TTTTTTGGATCCAGTAAAACCTGGCATGTA	pUC19 $P_{yjiY\ -212/+88}$
yjiY-5P-3 sense	TTTTTTGAATTCCGCCGAGTGAATTTTATTCA	pUC19 $P_{yjiY\ -212/+88}$
In vivo reporter		
upstream-replacement as	GGCTGGACTTCCGTCATGACGCGACAATTATTC	pRS415 $P_{yhjX\ up\_rplmt}$
upstream-replacement s	GACGGAAGTCCAGCCGGCATTTCATTCCGTTCT	pRS415 $P_{yhjX\ up\_rplmt}$
motif 1 replacement as	CGTCCCGTAATTAGTTCAGGAATGAATG	pRS415 $P_{yhjX\ M1}$
motif 1 replacement s	TTACGGGACGTCCGTTCTGATGGCATTT	pRS415 $P_{yhjX\ M1}$
spacer replacement as	CGACTCCATTCATGAAATGCCTTAGTTCA	pRS415 $P_{yhjX\ spacer}$
spacer replacement s	GAATGGAGTCGGGCATTTCATGCCGTTTT	pRS415 $P_{yhjX\ spacer}$
motif 2 replacement as	CGTCCCGTAAATCAGAACGGAATGAAAT	pRS415 $P_{yhjX\ M2}$
motif 2 replacement s	TTACGGGACGGCCGTTTTTCCCCAGGCA	pRS415 $P_{yhjX\ M2}$
downstream-replacement as	AGTTTTCCCCCATTAATGAAATGCCATCAGAAC	pRS415 $P_{yhjX\ down\_rplmt}$
downstream-replacement s	TAATGGGGGAAAACTGCATAAAGTGCACTTCGT	pRS415 $P_{yhjX\ down\_rplmt}$
motif shortening 1 as	ATCAGAACGGACTGAAATGCATTAGTTCAGGAATGAATG	pRS415 $P_{yhjX\ M2\ G/T}$
motif shortening 2 as	ATCAGAACGGACGGAAATGAATTAGTTCAGGAATGAATG	pRS415 $P_{yhjX\ M2\ GG/AT}$
motif shortening 3 as	ATCAGAACGGACGTAAATTAATTAGTTCAGGAATGAATG	pRS415 $P_{yhjX\ M2\ GGC/CAT}$
motif shortening 1 s	TCCGTTCTGATTGCATTTCAGGCCGTTTTTCCCCAGGCA	pRS415 $P_{yhjX\ M2\ G/T}$
motif shortening 2 s	TCCGTTCTGATTTCATTTCCGGCCGTTTTTCCCCAGGCA	pRS415 $P_{yhjX\ M2\ GG/AT}$
motif shortening 3 s	TCCGTTCTGATTTAATTTACGGCCGTTTTTCCCCAGGCA	pRS415 $P_{yhjX\ M2\ GGC/CAT}$

Table S3: Influence of C-sources and additives on *yhjX* expression. Strain and cultivation conditions were the same as described in Figure 4.4B.

Medium	Additional C-source	Concentration C-source	Additive	Concentration additive	Average of max. *yhjX* expression [RLU/OD_{600}]	Standard deviation of max. *yhjX* expression [RLU/OD_{600}]
LB medium	Glucose	0.4%	Pyruvate	20 mM	41,710	3,020
LB medium	Pyruvate	20 mM			38,810	1,270
LB medium	-	-	-	-	35,450	3,670
LB medium	Glucose	0.4%	-	-	10,820	1,910
M9 medium	Pyruvate	20 mM	-	-	479,070	46,060
M9 medium	Yeast extract	0.5%	-	-	38,240	4,460
M9 medium	Gluconic acid	0.4%	-	-	7,260	620
M9 medium	Glucuronic acid	0.4%	-	-	4,120	860
M9 medium	Lactate	20 mM	-	-	1,000	160
M9 medium	Phosphoenol pyruvate	20 mM	-	-	370	80
M9 medium	Glycerol	0.4%	-	-	360	70
M9 medium	L-Serine	20 mM	-	-	360	50
M9 medium	Acetate	0.4%	-	-	320	30
M9 medium	Lactose	0.4%	-	-	300	20
M9 medium	Casamino acids	0.4%	-	-	260	30
M9 medium	L-Proline	20 mM	-	-	260	40
M9 medium	Fumarate	20 mM	-	-	250	50
M9 medium	Mannose	0.4%	-	-	250	50
M9 medium	Succinate	0.4%	-	-	250	40
M9 medium	Galactose	0.4%	-	-	230	30
M9 medium	Oxaloacetate	20 mM	-	-	230	30
M9 medium	Peptone	0.4%	-	-	220	60
M9 medium	L-Asparte	20 mM	-	-	210	40
M9 medium	Mannitol	0.4%	-	-	210	40
M9 medium	Fructose	0.4%	-	-	200	40
M9 medium	Xylose	0.4%	-	-	200	50
M9 medium	Maltose	0.4%	-	-	170	40
M9 medium	Glucose	0.4%	-	-	160	150
M9 medium	L-Glutamate	0.4%	-	-	130	40
M9 medium	Tryptone	0.4%	-	-	10	10
M9 medium	Cas amino acids	0.4%	Glucose	0.4%	22,640	520
M9 medium	Cas amino acids	1.5%	PIPES (pH 5.5) + Glycerol	20 mM + 0.8%	9,910	720
M9 medium	Cas amino acids	1.5%	PIPES (pH 7.0) + Glycerol	20 mM + 0.8%	7,620	240
M9 medium	Pyruvate	20 mM	Fumarate	20 mM	528,780	66,840
M9 medium	Pyruvate	20 mM	Glucose	20 mM	281,270	28,830
M9 medium	Pyruvate	20 mM	Glucose	1 mM	270,880	41,130
M9 medium	Pyruvate	20 mM	Lactate + PIPES (pH 7.0)	20 mM + 20 mM	240,000	16,200
M9 medium	Pyruvate	20 mM	Glucose	5 mM	201,370	6,160

M9 medium	Pyruvate	20 mM	Acetate	20 mM	165,180	20,310
M9 medium	Succinate	0.4%	Pyruvate	20 mM	274,060	17,380
M9 medium	Succinate	0.4%	Fumarate	20 mM	9,940	1,130
M9 medium	Succinate	0.4%	Lactose	0.4%	3,350	790
M9 medium	Succinate	0.4%	Guanidine hydrochloride	1 mg/ml	660	390
M9 medium	Succinate	0.4%	Methanol	1%	600	480
M9 medium	Succinate	0.4%	Fosfomycin	1 μg/ml	570	480
M9 medium	Succinate	0.4%	Crystal violet	0.05 μg/μl	560	450
M9 medium	Succinate	0.4%	D-Leucine	20 mM	540	40
M9 medium	Succinate	0.4%	L-Histidine	20 mM	530	380
M9 medium	Succinate	0.4%	Sulfamethazine	0.5 μg/ml	510	30
M9 medium	Succinate	0.4%	Arsenate	20 μg/ml	500	190
M9 medium	Succinate	0.4%	Imipenem	0.05 μg/ml	480	210
M9 medium	Succinate	0.4%	Plumbagin	8 μg/ml	470	240
M9 medium	Succinate	0.4%	D-Argine	20 mM	410	430
M9 medium	Succinate	0.4%	D-Proline	20 mM	410	180
M9 medium	Succinate	0.4%	Maltose	0.4%	390	60
M9 medium	Succinate	0.4%	Xylose	0.4%	380	50
M9 medium	Succinate	0.4%	Antimony(III)chloride	5 μg/ml	370	140
M9 medium	Succinate	0.4%	Hdroxyurea	100 μg/ml	350	200
M9 medium	Succinate	0.4%	Paromomycin	0.01 μg/ml	350	150
M9 medium	Succinate	0.4%	Paromomycin	0.005 μg/ml	340	130
M9 medium	Succinate	0.4%	5,7-Dichloro-8-hydroxyquinaldine	1 μg/ml	330	360
M9 medium	Succinate	0.4%	D-Tyrosine	20 mM	330	110
M9 medium	Succinate	0.4%	Oxalate	30 mM	330	220
M9 medium	Succinate	0.4%	Propanol	1%	330	330
M9 medium	Succinate	0.4%	Thiamphenicol	1 μg/ml	300	160
M9 medium	Succinate	0.4%	Polymyxin B	0.005μg/ml	290	130
M9 medium	Succinate	0.4%	Deoxycholate	100 μg/ml	280	340
M9 medium	Succinate	0.4%	Lactulose	30 mM	280	250
M9 medium	Succinate	0.4%	Natriumphosphat (pH 7)	200 mM	280	250
M9 medium	Succinate	0.4%	7-Hydoxycoumarin	10μg/ml	270	220
M9 medium	Succinate	0.4%	Tobramycin	0.01 μg/ml	270	460
M9 medium	Succinate	0.4%	Arsenite	10 μg/ml	260	10
M9 medium	Succinate	0.4%	L-Alanine	20 mM	260	60
M9 medium	Succinate	0.4%	Iodacetic acid	10 μg/ml	250	240
M9 medium	Succinate	0.4%	L-Leucine	20 mM	240	230
M9 medium	Succinate	0.4%	Lactate	20 mM	230	90
M9 medium	Succinate	0.4%	Deoxycholate	50 μg/ml	210	220
M9 medium	Succinate	0.4%	L-Arginine	20 mM	210	180
M9 medium	Succinate	0.4%	L-Isoleucine	20 mM	200	40
M9 medium	Succinate	0.4%	L-Tyrosine	20 mM	200	190
M9 medium	Succinate	0.4%	N-Acetyl-Glucosamine	0.4%	170	40
M9 medium	Succinate	0.4%	Deoxycholate	500 μg/ml	160	280

M9 medium	Succinate	0.4%	NaCl	2%	160	40
M9 medium	Succinate	0.4%	PIPES (pH 7.0) + Oxalate	20 mM + 30 mM	160	40
M9 medium	Succinate	0.4%	Apramycin	0.005 µg/ml	150	140
M9 medium	Succinate	0.4%	Benzoate	30 mM	140	120
M9 medium	Succinate	0.4%	Ethanol	5%	130	230
M9 medium	Succinate	0.4%	Peptidoglycan E. coli	1/20 fold dilution	130	100
M9 medium	Succinate	0.4%	PIPES (pH 7.0) + Mitomycin	20 mM + 0.3 µg/ml	130	30
M9 medium	Succinate	0.4%	D-Cycloserine	0,5 µg/ml	120	190
M9 medium	Succinate	0.4%	D-Serine	20 mM	120	110
M9 medium	Succinate	0.4%	Ethanol	1%	120	200
M9 medium	Succinate	0.4%	Gly-Gly	20 mM	100	300
M9 medium	Succinate	0.4%	L-Asparagine	20 mM	100	140
M9 medium	Succinate	0.4%	L-Threonine	20 mM	100	180
M9 medium	Succinate	0.4%	PIPES (pH 7.0)	20 mM	90	20
M9 medium	Succinate	0.4%	D-Alanine	20 mM	80	130
M9 medium	Succinate	0.4%	L-Glutamate	20 mM	80	130
M9 medium	Succinate	0.4%	L-Proline	20 mM	80	140
M9 medium	Succinate	0.4%	Chlorambucil	50 µg/ml	70	120
M9 medium	Succinate	0.4%	Ethanol	2%	70	110
M9 medium	Succinate	0.4%	L-Glycine	20 mM	70	120
M9 medium	Succinate	0.4%	Phenyl-methylsufonyl-fluorid	200 µg/ml	60	110
M9 medium	Succinate	0.4%	PIPES (pH 7.0) + Formate	20 mM + 30 mM	60	20
M9 medium	Succinate	0.4%	L-Serine	20 mM	50	80
M9 medium	Succinate	0.4%	Peptidoglycan Bacillus	1/20 fold dilution	50	30
M9 medium	Succinate	0.4%	Peptidoglycan Lactobacillus	1/20 fold dilution	50	90
M9 medium	Succinate	0.4%	Amitriptylin	10 µg/ml	40	40
M9 medium	Succinate	0.4%	Formate	30 mM	40	70
M9 medium	Succinate	0.4%	L-Aspartate	20 mM	40	30
M9 medium	Succinate	0.4%	Methylglyoxal	0,7 mM	40	10
M9 medium	Succinate	0.4%	Peptidoglycan Lactobacillus	1/2000 fold dilution	40	20
M9 medium	Succinate	0.4%	D-Ala-D-Ala	20 mM	30	20
M9 medium	Succinate	0.4%	Dulcitol	0.4%	30	50
M9 medium	Succinate	0.4%	Methylglyoxal	0,2 mM	30	10
M9 medium	Succinate	0.4%	Peptidoglycan Bacillus	1/2000 fold dilution	30	40
M9 medium	Succinate	0.4%	PIPES (pH 7.0) + Benzoate	20 mM + 30 mM	20	10

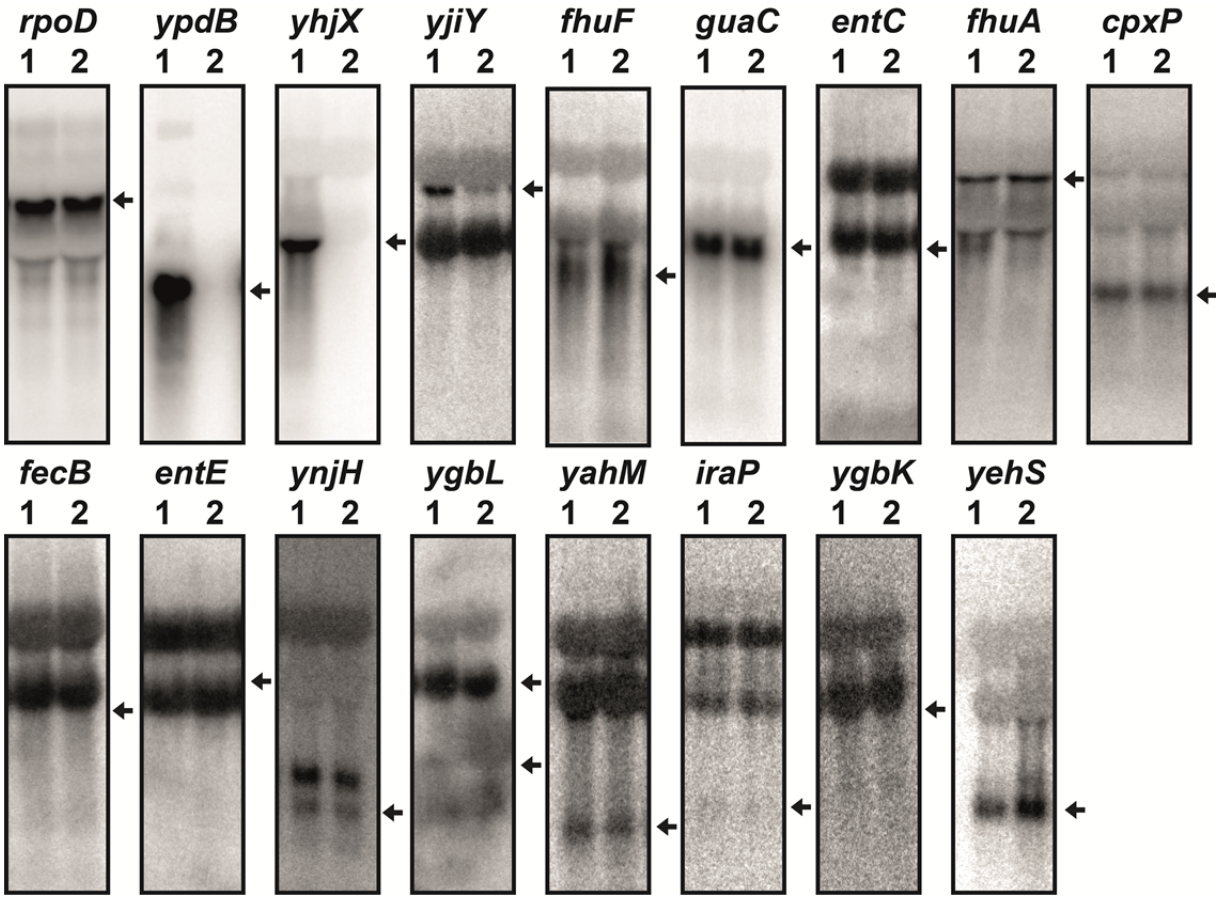

Fig. S1. Evaluation of potential YpdB target genes. A) Northern blot analysis was used to measure the effect of overproduction of YpdB on the expression of the genes identified by transcriptome analysis (see Table 4.1) and *rpoD* (control) in *E. coli* MG21 (Δ*ypdB*). The expression levels of these genes were also assessed in the *E. coli* strain MG21 (Δ*ypdB*) in the absence of YpdB (*E. coli* MG21 transformed with the empty pBAD24 vector) (lanes 2) or upon overproduction of YpdB (lanes 1). 20 µg of total RNA was loaded per lane, and the transcripts were detected with the corresponding gene-specific DNA probes. Transcripts of the corresponding genes are marked by an arrow.

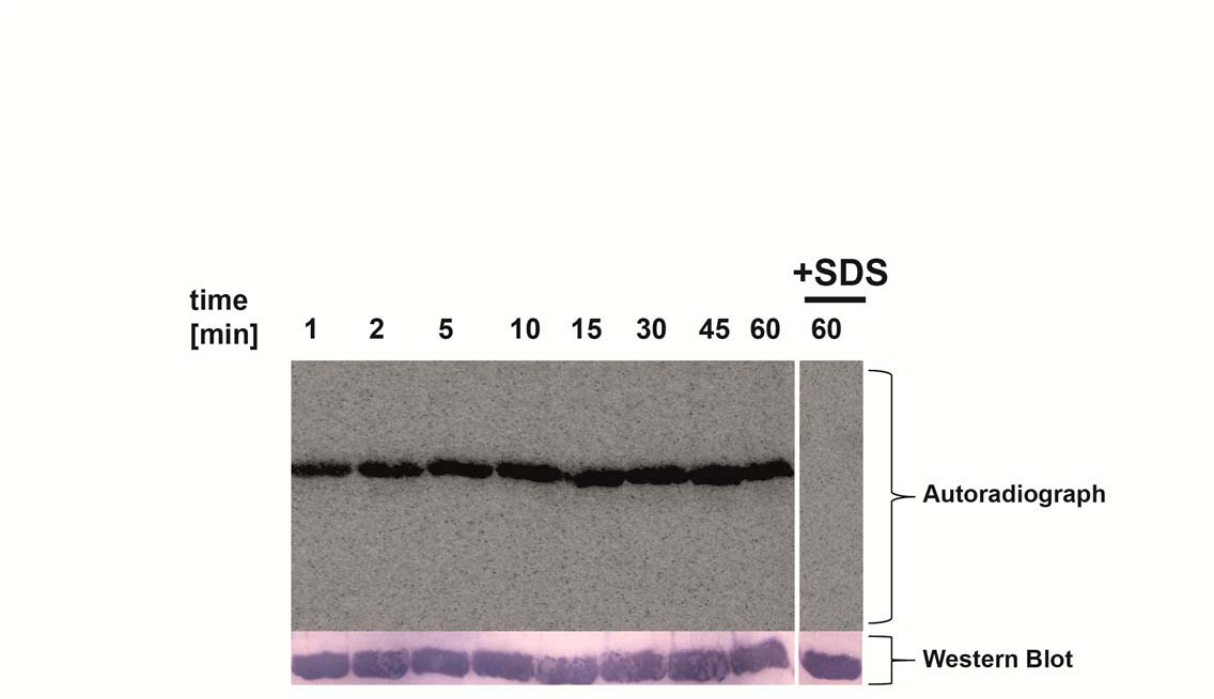

Fig. S2. In vitro phosphorylation of YpdB. Purified YpdB-6His was mixed with phosphorylation buffer. Phosphorylation was started by adding a mixture of [γ–^{32}P]acetyl phosphate and $MgCl_2$. At the indicated times, the reaction was stopped by adding SDS-sample buffer, the samples were subjected to SDS-PAGE and Semi Dry Western Blotting. As negative control, protein was denatured by adding SDS-sample before the reaction was started (+SDS). Phosphorylated YpdB was detected by autoradiography using a phosphor screen and a PhosphorImager Storm. The autoradiograph is representative of three independent experiments.

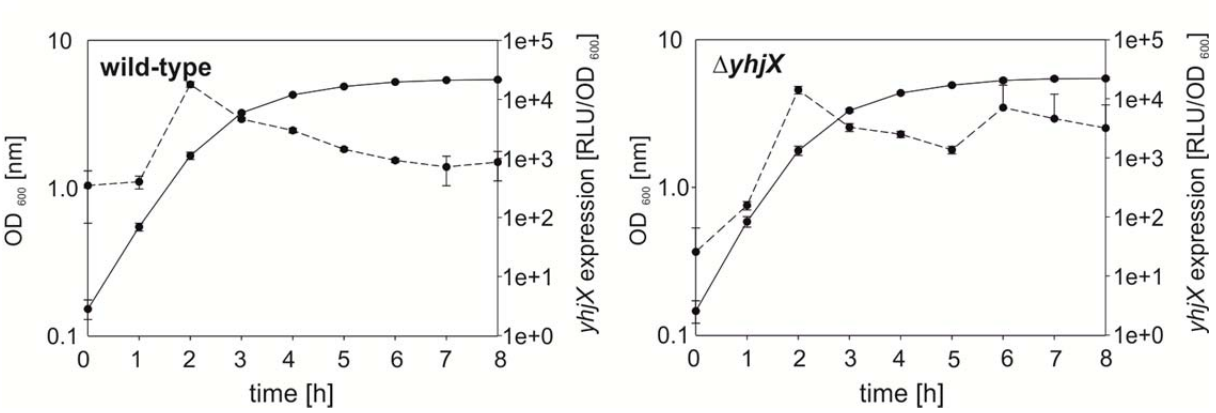

Fig. S3. *yhjX* induction is independent of YhjX feedback regulation. *Escherichia coli* MG1665 (wild-type) and MG26 (Δ*yhjX*) were transformed with pBBR *yhjX*-lux and grown aerobically in LB medium. Growth and luciferase activity were monitored continuously. The maximal luciferase activity normalized to an optical density of 1 (RLU/OD600) was used as a measure of the degree of induction of *yhjX*. Data were obtained from at least three independent experiments, and average values were used for calculations.

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Supplemental Material – Chapter 5

Supplemental Material:

Identification of the LytS/LytTR-like signaling network in *Escherichia coli*

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Running title: the LytS/LytTR network in *E. coli*

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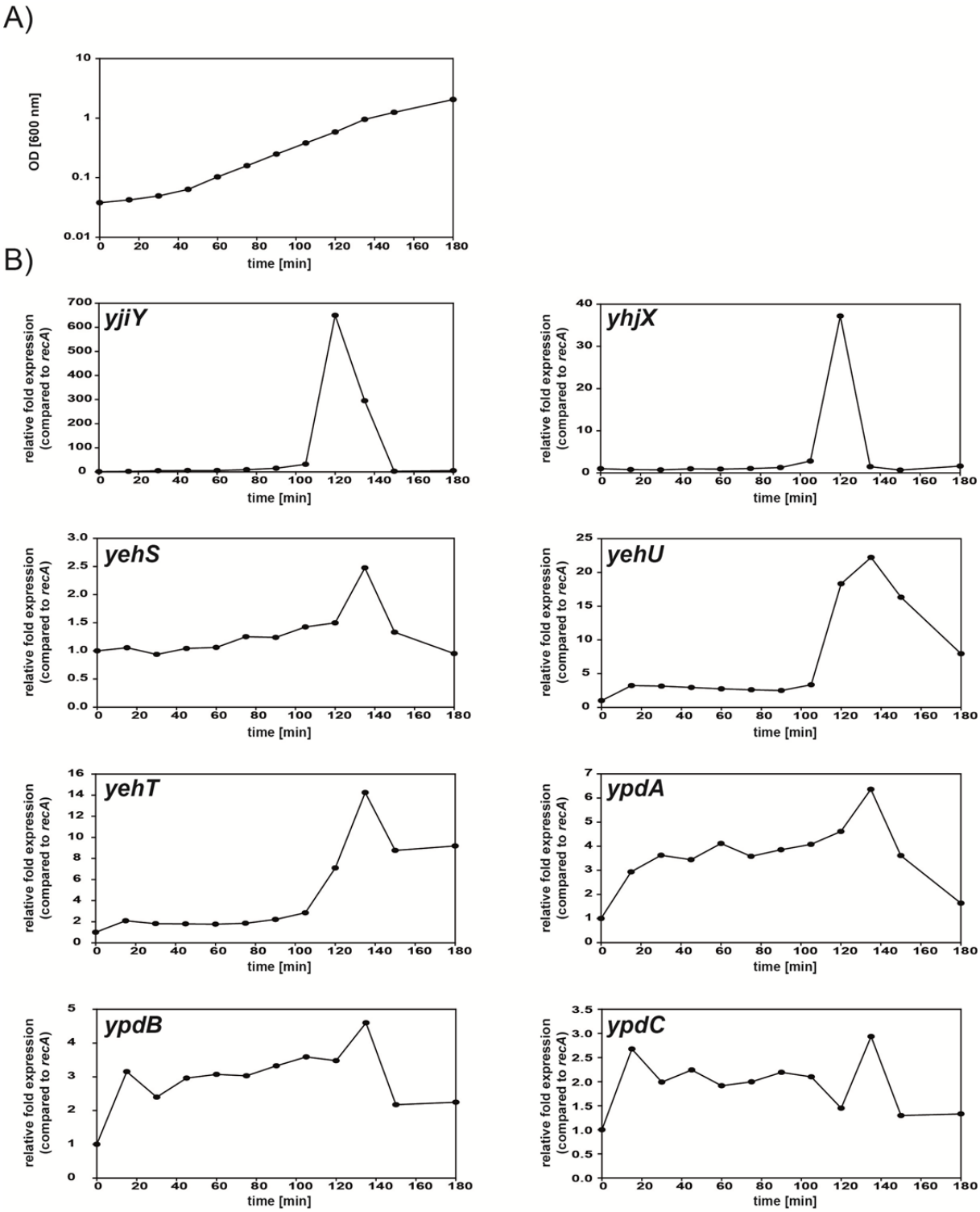

Fig. S1. Transcriptional analysis of the LytS/LytTR-like two-component system, target genes *yhjX*, *yjiY* and the associated gene *yehS*. Cells of the wild type (MG1655) were shifted from a stationary phase (stat) culture of M9 medium with Glucose as C-soure in LB medium and grown (A) as described in Material & Methods. Total RNA was isolated at different time points (marked by the crosses) in all growth phases and cDNA synthesized. Levels of *yjiY*, *yhjX*, *yehS, yehU, yehT, ypdA, ypdB, ypdC* and *recA* (as reference) transcripts were determined by qRT-PCR for each time point. Changes in transcript levels (expressed relative to *recA*) were calculated using the C_T method. Relative transcript levels were normalized to 0 min values. All experiments were performed in triplicate and mean values are shown, the standard deviations were below 15 %.

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